STUDIES ON PROLIFERATION IN SUNFLOWER TISSUES INDUCED BY *ESCHERICHIA COLI*

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E. L. FENWICK

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ABSTRACT

An investigation has been carried out on the effects of inoculating sunflower tissues with Escherichia coli following Philipson and Sheat's (1963) report of the stimulatory action of this bacterium on sunflower hypocotyl tissue.

No consistent results could be obtained when aseptically grown decapitated seedlings were inoculated with E. coli, so that in most experiments 1 mm-thick disks of sunflower hypocotyl were grown on a simple sucrose-inorganic salts medium which was modified on occasion with the addition of indole acetic acid. Disks cultured in this way were inoculated with bacteria and grown in the light or darkness at 25°C. After treatment observations were made of the gross morphology of the disks while fresh and dry weights were recorded. In addition a detailed histological study was carried out. The reactions to inoculation of older sunflower plants and of clonal pith tissue were also investigated.

Hypocotyl disks inoculated with E. coli and grown in the light proliferated from the lower surface and formed numerous long roots while those in the dark were usually inhibited in comparison with uninfected disks. Inoculation of disks with the crown gall bacterium, Agrobacterium tumefaciens, induced proliferation mainly from the upper surface and a few short roots were formed. Although the addition of 0.01 ppm IAA to uninfected disks induced growth similar to that in E. coli-inoculated disks grown in the light on the simple medium,

histological studies showed that the proliferating tissues were different in character. The histological studies also showed distinct differences between the proliferation in E. coli and A. tumefaciens-infected disks. By increasing the time between wounding and inoculation with E. coli the growth response of the disks was shown to be closely connected with wounding.

Since E. coli can produce IAA and vitamins under certain culture conditions, and other bacteria have been shown to produce cytokinins, it was suggested that the bacteria produce some substances of this nature reacting with freshly-wounded tissue, which induce proliferation in light-grown disks or inhibition in disks grown in the dark.

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CHAPTER ONE

FACTORS RESPONSIBLE FOR ABNORMAL GROWTH

The way in which plants develop depends on their genetic constitution and the environment in which they are grown. When plants with like gene complements are grown under similar environmental conditions they tend to develop in the same way, although there are small differences between them. Moreover, when such plants are subjected to external influences not usually present, they may react in a specific way by producing abnormal growth forms. Sinnott (1960) defines abnormal growth as the production of some structure which does not comform to the normal pattern of growth. The tissues in these structures do not differ from those found in normal tissue, although their arrangement in relation to one another may be different (Bloch 1941). However, many gradations occur between obviously normal and distinctly abnormal growth, so that the two cannot be separated absolutely; the structures formed range from simple unorganized intumescences to highly organized galls consisting of up to five different types of tissue.

This review is concerned with the various ways in which relatively unorganized forms of abnormal growth are induced in higher plants, and with the physiology of these tissues. The subject of this thesis concerns the nature of the unorganized tissues formed under the influence of the bacterium Escherichia coli (Migula) Castellani and Chalmers. Since this has not been reported previously, a comparative review of the range of abnormal growth forms is necessary, in order to determine whether E. coli acts in a similar manner to any other agent in inducing tissue proliferation. Many

of the forms of abnormal growth described below have been reviewed recently (Klein 1965, Braun and Stonier 1958, Black 1965, Maresquelle and Meyer 1965, Kehr 1965), so no attempt is made to cover this wide field in detail.

Agents capable of inducing unorganized abnormal growth in higher plants include physical agents such as water or wind, chemical agents ranging from simple inorganic compounds to complex growth substances, bacteria, viruses, fungi, insects, and genetic factors. Most of the proliferation induced by such agents is self-limiting so that proliferation ceases when the agent is removed from the plant. Such abnormal growth is also limited by the extent of response the tissues are able to make to the agents. Non-limited proliferation is the result of uncontrolled and unlimited growth on which the plant has no influence (White 1951). The main example of this autonomous growth is crown gall, in which cells become permanently altered by the presence of the bacterium Agrobacterium tumefaciens (Smith and Townshend) Conn, and continue their abnormal growth after the bacteria have been removed from the tissues. Only two other types of abnormal growth can be regarded as truly autonomous, namely the genetic tumours of Nicotiana hybrids and Gautheret's habituated tissue. Although Braun and Stonier (1958) regard the tumours caused by wound tumour virus as examples of non-limited proliferation, the virus has not yet been removed from the proliferating tissues, and growth cannot yet be classified as autonomous.

Tissue culture represents a special case of plant proliferation. Although growth is unorganized it can hardly be described as abnormal, since it is the normal expression of the tissues when separated from

the restraining influences of the plant as a whole (Sinnott 1960). However, tissue culture of abnormal growth forms, in particular crowngall tissues, has proved to be an excellent means of studying changes in metabolism.

Characteristics of the different forms of unorganized abnormal growth, grouped according to the agents by which they are induced, will now be considered in more detail. The morphology and histology of such structures are described in Chapter 8.

1.1 Physical Agents.

One of the commonest physical agents causing abnormal growth is mechanical damage in the form of wounding. Plants respond to this by the formation of a layer of cork cells or by the production of callus near the wounded surface. Although these tissues occur frequently as the result of injury they are not usually found in normal whole plants and because of this their growth has been termed abnormal (Sinnott 1960).

It appears that cell growth near a wound is influenced by some active substance which has been called a wound hormone. This was demonstrated by Haberlandt (1913) with kohlrabi tissues. He showed that outgrowths formed on cut stems if the wound were left untouched or if it were washed and then covered with crushed parenchyma. No outgrowths appeared when the wound was washed without further treatment.

There have been numerous investigations into the nature of the wound hormone. Bonner and English (1938) found that traumatin, a dibasic acid isolated from Vicia faba, was effective in stimulating cell division in bean pods. They emphasized that this substance

was unlikely to be a universal wound hormone. Hemberg (1943) reported that in cut disks or slices of potato tubers two kinds of growth substances were found after the pieces had been exposed to the air for three hours. One of these was probably indole acetic acid (IAA), and it was thought that the second substance could be indole acetaldehyde. Indirect confirmation of the presence of auxins near wounded cells came from the observation of Brown (1937) that wound cambial activity was promoted in Populus balsamifera by the presence of developing leaves distal to the wound. These are known to be a source of auxin. In addition, increased respiration and protoplasmic streaming in wounded cells are thought to indicate the existence of an auxin type of stimulation (Fitting 1925). On the other hand Thimann (1960) has reported that auxin is destroyed by wounded tissues. It is generally agreed that whether substances active in inducing cell division in the vicinity of the wound are auxins or not, they are probably degeneration products of cells which necrose near the wound. It often appears as if the type of response depends simply on the concentration of wound substance, although there is no quantitative evidence of this (Bloch 1953).

Besides the action of degenerative substances, external physical conditions are also important in determining the response of tissue to wounding. These conditions include temperature, humidity, light and access to oxygen. La Rue (1937) found that under moist atmospheric conditions, cells at the exposed surfaces hypertrophied, whereas the usual pattern of wound-healing involved the deposition of a suberised layer. Priestly and Woffenden (1922) found that when

access to oxygen was cut off, cells in the vicinity of the wound slowly developed outgrowths similar to intumescences: intumescences arise in localized areas usually as a result of cell expansion rather than division. Bloch (1937) concluded that in addition to the active wound substances, oxygen and high water content affected both wound healing and necrosis.

It has been stressed by Bloch (1952) that a thorough knowledge of the various types of wound callus is essential before a valid interpretation can be made of structures induced by the application of growth substances, or various pathogens. In all plants, there are only a limited number of cell types which can react. Consequently the range of morphological variation is not large, and great care is required in attributing any differences from wounding to further abnormal growth.

Certain physical effects not involving wounding of the tissues have been found to stimulate abnormal growth. La Rue (1933) showed that intumescences were formed on poplar leaves when they were kept in a moist atmosphere. In onion roots, an applied electric current was found to cause abnormal growth (Berry, Gardiner and Gilmartin 1947). At certain levels of intensity the current produced gross abnormalities in the roots, similar in appearance to tissues treated with growth substances. In this case it was apparent that the growth hormonal system in the roots had been disrupted by the flow of the electric current.

Although radiation in the form of X, γ or β rays is known to affect plant growth in numerous ways, few reports have been made of tissue stimulation occurring as a direct result of radiation

(Gunckel 1965). Adventitious meristems and tumours were induced in Ipomoea tuba by Biddulph and Biddulph (1953), and in tobacco hybrids by Kehr and Smith (1954). In the latter case, however, it is known that tobacco hybrids often form tumours when wounded, and they may have resulted from radiation damage to tissues. More usually, radiation causes stunting of growth due to a reduction in auxin levels (Gunckel and Sparrow 1954).

A number of simple chemical compounds have been found to stimulate abnormal growth, but this is effected primarily through damage to the tissues. Although ethylene gas occurs naturally in some plants, it is active in relatively high concentrations (1% by volume) in intumescence formation through its corrosive action on the cell walls, which is accompanied by great cell hypertrophy (Wallace 1928). Young potato plants treated with copper compounds form intumescences (La Rue 1933), again presumably through chemical damage to the cells. However, stimulation of meristematic tissue formation in potato plants by the application of low concentrations of zinc sulphate (Silberberg 1909) is apparently not accompanied by wounding.

Numerous complex organic compounds, carcinogenic in animals, were applied to plants by Levine (1934, 1940, 1942, 1950). The materials tested included coal-tar derivatives. Levine (1942) found that the reaction of the plant tissues to the substances was more a function of the tissues themselves than of the chemicals applied. The plants showed a protective response which was thought to be greater than the reaction of the tissues to mechanical injury (Levine 1934), but autonomous growth could not be induced

with any of these compounds.

1.2 Growth Substances.

There are many chemical compounds of widely differing structure which are very important in influencing growth and development through their physiological activity (Sinnott 1960). These contrast with such compounds as have already been described in that very small amounts are required to influence fundamental cell processes. Growth substances, in combination with the genetic constitution of the plant, seem to be the main agents controlling growth and development (Sinnott 1960). Although they affect such diverse plant activities as tropisms and the differentiation of specific organs, only their activities in causing abnormal undifferentiated growth will be considered in this review.

The most intensively studied group of growth substances is the auxins. Although IAA is the most widely-known naturally occurring auxin there are many other compounds, some chemically related, with the same or similar effects on plant tissues. These, however, do not occur naturally in plants and will be referred to as synthetic auxins (Audus 1959). Brown and Gardner (1936) were the first to observe that application of IAA could induce overgrowths and tumours in red kidney bean plants. Since that time, many different species of plants have been treated in the same way. The formation of these overgrowths has been studied mainly from a histological point of view (Kraus, Brown and Hamner 1936, Borthwick, Hamner and Parker 1937 Hamner and Kraus 1937). In most cases IAA was applied in a lanolin paste to the cut surface of decapitated stems and the callus produced was in the form of a rounded dome-shaped mass from which

root primordia emerged. In red kidney bean, the overgrowths were likened to crown galls by Kraus et al (1936). Generally the response of different plants to IAA was similar although variations in the extent of reaction occurred among the different species (Borthwick et al 1937, Palser 1942). Hamner and Kraus (1937) found that overgrowths and root formation were also induced when IAA was applied to stems which had not been wounded.

In addition to IAA, the effects of synthetic auxins on the growth of plants have been studied (Blum 1941, Swanson 1946, Hamner and Kraus 1937). As with IAA, callus formation generally resulted although the amount formed depended on the nature of the auxin and the species of plant used. These substances differed from IAA in the degre to which root formation was induced in the callus. Tryptophane, which can be readily transformed to IAA (Fallot 1964) was applied to decapitated bean plants by Kraus (1941). The apical tumour formed was different in shape from that induced by IAA because the pith tissues did not proliferate.

For detailed information on the mode of action of auxins in stimulating tissue proliferation, small segments or tissue cultures of plants have been grown on chemically defined media. In this way many of the variables present in whole plants are eliminated so that it is possible to study the direct action of the auxins on the tissues. The first work of this nature was carried out by Beal (1940), using sections 0.5 mm thick, or detached segments 5 mm long of the second internode of Phaseolus vulgaris. Where IAA was applied, the tissues were stimulated to divide and sugar and nitrogenous substances moved to the area. In this way the dividing

cells were supplied with materials necessary for growth. De Ropp (1947a) studied the effects of IAA, indole butyric and naphthalene acetic acids (NAA) on 3 mm long pieces of sunflower stem tissue from which the epidermis had been stripped. Roots were induced to form with low concentrations of these auxins (0.01 ppm). The cambium proliferated with higher concentrations and root formation was inhibited with concentrations of 10 ppm. Variations in the extent of the response occurred among different auxins. In their study of 35 mm segments of the pea epicotyl, Sorokin, Mathur and Thimann (1962) concluded that IAA caused activation of the cambium, producing abnormal growth.

Most of the work on auxin-stimulation of growth has been carried out on tissue cultures rather than on stem segments. These cultures are usually composed of only one tissue type although combinations of two or more tissues may be used. With these tissues it is difficult to distinguish what constitutes abnormal proliferation. It has been found that auxins are very important in maintaining normal growth of tissues in culture, by inducing cell division (Gautheret 1959). A number of tissues now grown in culture are dependent on a supply of auxin, and growth ceases in its absence. For different tissues the amounts of auxin required vary, and the type of growth which is induced is dependent on the concentration of auxin in the medium (Gautheret 1959). With low concentrations, tissues show a stimulation of cell division. With increasing concentrations inhibition of bud formation may occur in tissues which formerly produced them, while rooting is increased and stimulation or inhibition of leaf growth occurs. Finally, with still higher concentrations, hyperhydric transformation takes

place; hyperhydric tissues are characterized by increased cell enlargement and water uptake, and little cell division occurs. Not all these reactions are shown by any one tissue.

These effects of differing auxin concentration are not usually permanent since the growth pattern frequently changes when the tissues are subsequently cultured on different media (de Ropp 1947a). However, a form of abnormal growth, similar to the autonomous growth of crown gall tissues occurs in a limited number of plant tissues which normally require auxin for growth, but which become independent of it. This adaptation of tissues to growth without auxin was first described in carrot tissues by Gautheret in 1946. It has been called 'accoutumance' or 'anergie' by the French, but has more recently been termed 'habituation' by American authors (Braun and Stonier 1958).

Habituation usually occurs when certain tissues such as those of carrot, Scorzonera, vine, tobacco, mallow, sweet clover or sunflower, have undergone numerous transfers on media containing auxins. The conditions under which the tissues are grown are important in causing their transformation. High concentrations of auxin (either IAA or NAA) are more effective than low concentrations. More cultures become habituated at temperatures of 24° to 27°C than 33° to 36°C since growth at the latter temperature range is not as vigorous (Gautheret 1959). With some types of tissue habituation occurs frequently while others seldom or never undergo the change. Habituated tissues become transparent and friable and only a small amount of differentiation is apparent. No roots are ever formed from such tissues. Tumours may be obtained when habituated tissues

are grafted to normal stems (Camus and Gautheret 1948a and b, Limasset and Gautheret 1950). In some cases, the tissues do not become fully autonomous although their requirements for auxin are much lower than unchanged tissues. In certain cases, for example in carrot or Scorzonera, it has been found that habituated tissues may have a tendency to return to the normal state, with a decrease in growth and loss of friability of the tissues. However, the return is not complete since the tissues remain unable to form roots (Gautheret 1946).

It has been found that habituated tissues differ from normal tissues in their composition. Lee (1952) showed that concentrations of total and soluble nitrogen were higher in habituated tissues than in normal ones. Also, the concentration of free auxin was higher in the altered tissues (Kulescha and Gautheret 1948).

The nature of the change occurring in tissue cultures, bringing about habituation, is not known but Gautheret (1955) surmises that the chemistry of these cells is greatly modified. He has interpreted this to be a kind of enzymatic adaptation rather than mutation or selection of particular cells.

In most forms of abnormal growth, including wounding, auxin has been implicated as taking a major part in the cellular changes occurring, but whether it is a causal agent or merely a result of the changes is not known. The activity of auxin in these other forms of growth will be discussed in later sections.

In addition to auxins, it has been shown that gibberellins and cytokinins are active as growth substances but their role in abnormal growth has not yet been elucidated.

Although it has long been known that giberellins are produced by fungi, the first report of their isolation and identification from higher plants was made in 1958, when gibberellin A_1 was isolated from immature runner bean plants by MacMillan and Suter (1958). Sinnott (1960) in listing the properties of gibberellins, likens them to auxins, although he states that they are not able to initiate callus formation. However, in their review of this topic Phinney and West (1961) state that gibberellins do cause callus formation in tissue culture. Fallot (1964) has summarized the instances in which cell division is stimulated by gibberellin alone or in combination with auxins. It is apparent that the reactions of plant cells to gibberellins are dependent on their concentration and on the nature of the tissues to which they are applied. This was first clearly demonstrated by Nickell (1958), who showed that all types of response from inhibition to stimulation can be shown by different tissues treated with the same concentration of gibberellin. Using 10 ppm gibberellin, normal root callus of Melilotus officinalis was stimulated, while crown gall calluses of other plants were either stimulated or inhibited, according to the species. Wareing (1958) showed that callus was formed at the cut surface of stem pieces of sycamore, poplar and ash treated with gibberellic acid, but this was not as large as when IAA was applied alone or in combination with the gibberellin. Further to this work it was found that gibberellic acid alone, or in combination with IAA, promoted cell division in sycamore cambium tissues when applied at concentrations of 15 to 50 ppm although no increase in fresh weight was recorded with the highest concentration (Digby, Thomas

and Wareing 1964).

As with gibberellins, cytokinins occur in very small quantities in plant tissues, so that only one, zeatin, has so far been isolated from plant tissues (Letham 1963). However the effects of applied cytokinins on plant tissues are fundamental. Like gibberellins, their sphere of influence is very wide and their activities overlap with those of auxins so that they are also effective in promoting callusing in plant tissues (Thimann 1963). Wareing (1958) noted that the activity of gibberellin in sycamore was increased by the addition of IAA, and it is apparent that kinetin too acts synergistically with auxin in its effects on some tissues. In tobacco pith culture for example, Miller, Skoog, Okumura, von Saltza and Strong (1955) found that no cell division occurred in the presence of IAA or kinetin alone, but tissue proliferation was brought about when both substances were present.

Kinetin was shown to induce the formation of 'pseudo-nodules' on tobacco roots by Arora, Skoog and Allen (1959). Even more pronounced callusing occurred when petioles of dwarf beans with attached leaves, were grown in a solution containing 1 mg or 10 mg per litre of kinetin. In this case root formation was inhibited at the same time as callus formation was induced (Humphries 1960). In sunflower hypocotyl tissues de Ropp (1956) found that no abnormal growth was induced with kinetin although root formation was inhibited. Sorokin, Mathur and Thimann (1962) compared the growth of sections of pea epicotyl on media containing various auxins or kinetin and showed that callus was not induced by kinetin, while tissue growth was more normal than when auxins

were added. In tissue cultures of excised Helianthus tuberosus tissues, Nitsch and Nitsch (1957) found that kinetin induced cell division in tissues immersed in the medium, but not in those growing above this level. This action was very different from that observed for auxin, where all tissues on a fragment were induced to proliferate.

It is evident that, as with gibberellins, tissues show a variety of responses to kinins and that although growth substances mainly influence the normal growth of plants, they may under special conditions be responsible for a disruption of the normal tissue patterns thereby inducing abnormal growth.

1.3 Bacterial Agents

a) Crown gall induced by Agrobacterium tumefaciens. The most intensively studied plant gall is the crown gall caused by infection of tissues with the bacterium Agrobacterium tumefaciens. There has been so much interest in this topic that over 3,000 papers have been published (Klein 1965). However, for the purposes of this work several recent detailed reviews (Klein 1965, Braun 1962, 1954, Braun and Stonier 1958) have been used in conjunction with certain relevant papers.

The crown gall disease has received so much attention because the infected cells become altered in their reactivity so that they become self-regulating and are not subject to the influences of neighbouring plant tissues. The change is permanent and is not dependent on the continued presence of the bacteria. In this way crown gall tissues are similar to the habituated tissues described

in the previous section, although they show a higher degree of autonomy (Kulescha and Gautheret 1948, de Ropp 1951b).

Prior to 1936 most of the work on crown gall was concerned more with descriptions of its morphology and anatomy than with the physiological requirements of the tissues. When Kraus et al (1936) discovered that IAA could cause unregulated growth of cells similar to that found in crown gall, however, attention was directed to physiological aspects of the disease. A further stimulus was the development of suitable media for the growth of tissue cultures (White 1939), enabling the effects of the bacteria to be studied on certain selected tissues without the modifying influences of adjacent plant tissues. A few years later White and Braun (1942) showed that it was possible to grow crown gall tissues free from the bacteria, and much of the recent work has involved such tissues. Since 1942, studies on the physiology of crown gall formation have been mainly directed towards finding how the tissue becomes autonomous (Braun and Stonier 1958).

It was recognized early in research into this topic that for galls to be formed, the bacteria must enter the plants through fresh wounds (Braun and Stonier 1958). The numbers of bacteria introduced into the wound influenced the gall size to a limited extent, but several workers showed that the size of the gall was chiefly dependent on the severity of the wound (Smith 1922, Riker 1923, Levine 1923). It appeared that the amount of wound juice released from damaged cells was the most important factor. Klein (1954) verified this by showing that no galls were formed when the wounded tissue was washed before inoculation unless wound juice was supplied.

In addition, healed tissues would only form galls when wound juice was supplied (Klein 1954, 1955). Further evidence on the influence of wounding on subsequent gall formation was provided by Braun's study of tobacco pith cultures (Braun 1956, 1957a, b). No transformation to tumour cells occurred when such cultures were inoculated with A. tumefaciens. Histological studies showed that the tobacco pith cells were incapable of showing wound healing activity unless associated vascular tissue was present, when both wound healing and the initiation of tumours could occur. It was thought that the action of wounding influenced the cells by bringing them into a state of active division so that they became susceptible to transformation into tumour cells by the bacteria (Braun 1956, Braun and Stonier 1958). Klein (1954) considered that the actual wound substances may also be necessary for the formation by the bacteria of a substance which induces the change in the plant cells.

Braun (1947a, 1952) showed that for normal cells to be converted to tumour cells they must be conditioned by the wounding process and that this conditioning takes place gradually. In Kalanchoe daigremontiana and Vinca rosea conditioning reached a maximum 60 hours after wounding, declining later so that no tumours were formed when bacteria were applied five days after wounding. It was suggested that the time of greatest conditioning in the cells, 60 hours after wounding, was correlated with the time of the first large burst of mitotic activity in cells undergoing normal wound healing (Braun and Mandle 1948, Braun 1962). Using sunflower stem fragments de Ropp (1948a) found that the period required before maximum gall formation was different; cells remained in a condition to

react with the bacteria up to a week after they had been wounded although no gall formation resulted when the bacteria were applied two weeks after wounding. Braun (1954) reported that tomato tissues remained conditioned for two weeks after wounding. The different periods for which the tissues of different species remain susceptible to gall formation indicates that the conditioning phase in crown gall formation is not as strictly delimited as Braun implies. In this connection Klein (1965) stated that the time at which conditioning of the cells is greatest, is dependent on the test plant.

Through the wounding process then, the host cells are conditioned so that they are susceptible to bacterial induction of gall formation. This conditioning phase is independent of the presence of the bacteria.

Following the conditioning of the plant cells, tumour formation can be induced by inoculation with A. tumefaciens. Although conditioning of the cells apparently occurs at all temperatures at which plant growth is continued, gall induction occurs in some plants only below 30°C, although normal healthy growth of both the tissues and the bacteria is maintained at higher temperatures. This temperature effect was first demonstrated by Riker (1926). Braun (1947a) showed that the temperature inhibition of gall formation was connected with the induction and not the conditioning phase. He also showed (Braun 1957a) that like conditioning, induction was a time-dependent process, requiring 30 to 60 hours to go to completion.

These studies indicated that some factor was synthesized by the bacteria which acted on the conditioned cells (Klein 1965). Since the crown gall bacterium was known to synthesize IAA from tryptophane (Berthelot and Amoureux 1938), and crown galls are hyperauxinic (Kulescha and Gautheret 1948), it was suggested that crown gall formation was induced by the bacterial IAA. However, Riker, Henry and Duggar (1941) concluded that tumour induction was independent of bacterial IAA. Braun (1947b) called the active substance the tumour-inducing principle, and since the recognition of its action many workers have attempted to characterize it. The metabolic products of the bacteria were thought to be the source of the tumour-inducing principle. Several attempts have been made to obtain tumours with bacteria-free culture filtrates (Manil and Straszewski 1951, Bender and Brucker 1956, Klein 1954, Klein and Braun 1960), or with fractions of the bacteria (Manigault and Stoll 1958a, b, Manigault, Schaeffer and Stoll 1959). Proliferation was induced by the bacteria-free culture filtrates but Klein and Braun (1960) concluded that some bacteria must have passed through the filter so that the filtrates were not, in fact, bacteria-free. The results of experiments with the fractions of bacteria have been questioned by Braun and Stonier (1958), and tissue proliferation has not been successfully induced by the same means again. Braun made a critical evaluation of the experimental data relating to the tumour-inducing principle in 1962. It is apparent that although the tumour-inducing principle is formed in the presence of the bacteria, it is not a direct product of the bacteria in culture or a fraction of the bacterial cell.

In experiments investigating the deoxyribose-nucleic acid (DNA) content of tissue at the time of gall induction, Klein (1952) reported a sharp rise in DNA two days after inoculation. The DNA was reported to be due either to the host or to the bacterial cells (Klein, Rasch and Swift 1953), and less was accumulated at 30°C. The work was repeated by Kupila and Stern (1961) who found the same rise in DNA content in both wounded controls and in tissue which had been inoculated with bacteria. They concluded that the rise in DNA content was the product of shock in wounded tissues, so that it is not the first change in cells undergoing tumour induction.

A further interpretation of the nature of the tumour-inducing principle was suggested by de Ropp (1947b, 1951b) and has since been considered by a number of workers. He thought that the tumour-inducing principle could be a virus, since he found that crown gall tumours were formed in previously healthy tissues when bacteria-free tumour tissues were grafted onto normal sunflower stem segments (de Ropp 1947b). These results were also obtained by Camus and Gautheret (1948a), and by McEwen (1952). The work suggested that some substance moved from the original tumour tissues inducing proliferation in new tissues. De Ropp thought this to be a virus but McEwen felt that the tissue proliferation was probably caused by diffusion of auxin from the hyperauxinic tissues of crown gall. Black (1957) has discussed at length the evidence both for and against the tumour-inducing principle being a virus and concluded that failure to find the virus, or evidence of it, was not sufficient proof on which to exclude the possibility that the tumour-inducing principle was a virus.

The most recent study of the nature of the tumour-inducing principle is that carried out by Spurr, Hildebrandt and Riker (1962). They measured the levels of ascorbic acid oxidase and tyrosinase in normal and wounded tissues, as well as in those inoculated with A. tumefaciens, and found that the activities of these enzymes at 26° and 32°C increased to a peak two to four days after wounding although they later returned to normal. The levels of ascorbic acid oxidase and tyrosinase in tissues inoculated with A. tumefaciens remained high at 26°C, but at 32°C the levels in the inoculated tissues were the same as for wounded tissues, and they decreased to normal after sixteen days. Ascorbic acid oxidase and tyrosinase activities increased with time in inoculated tissues held at 26°C. Crown galls produced by attenuated strains of bacteria showed lower activities of the enzymes than fully altered tissues. Thus the enzymes showed the same positive and negative activities with temperature changes as were shown by conditioned cells when they became changed in tumour induction. Although the actual nature of the tumour-inducing principle is unknown the activities of these enzymes must be closely connected with the ability of cells to form crown galls.

Klein has postulated a third phase, that of promotion in the transformation of normal to tumour cells. Experiments using attenuated strains of bacteria showed that when auxins were applied fast-growing tumour cells could be produced from slowly growing cells which had been changed by the bacteria (Braun and Laskaris 1942, Thomas and Riker 1948). Klein and Link (1952) found that

the auxin must be applied after the conditioning and induction phase to be effective in forming large galls and that the growth capacity of the resulting tumours was dependent on the length of time the auxin was present after induction. It was found that antiauxins could prevent tumour formation when applied during the first four days after inoculation (de Ropp 1951b). Klein and Link (1955) concluded that some of the auxin which they thought necessary for the promotion phase was provided by the bacteria. This bacterial auxin has been termed a co-carcinogen. Skoog (1954) postulated that although attenuated and virulent strains of A. tumefaciens produce the same amount of auxin, the attenuated strains also produce an auxin inhibitor so that different amounts of auxin reach the plant tissues. This would explain the difference between galls formed from virulent and attenuated strains of bacteria.

Braun (1954) opposed this theory of the presence of a bacterial co-carcinogen and stated that the conditioning and induction phases were sufficient to change the normal cells into gall cells although attenuated bacteria alter them to a lesser extent than fully virulent strains. This assumption implied that the tumour-inducing principle produced by attenuated strains of A. tumefaciens was not as effective as that of virulent strains, and differed from it in some ways (Braun and Stonier 1958).

As a result of the transformation of normal cells to crown gall cells the tissues acquire new properties. The technique of tissue culture has greatly facilitated the study of the physiological properties of these tissues. The mineral requirements of

crown gall tissues in culture or in intact plants are not very different from those of normal tissues (Klein 1965). The tissues show increased quantities of phosphorus and nitrogen compounds due to the actively-dividing nature of the gall cells (Braun 1962).

Apart from some work showing the influence of auxins on intact plants (Braun and Laskaris 1942, Thomas and Riker 1948) most research on growth substances in gall tissues has been carried out on tissue cultures. These have been successful because of the ease with which bacteria-free tissues can be obtained. As a result of the changes which take place, crown gall tissues become autonomous in their requirements for growth substances, particularly auxin (Klein and Vogel 1956). In vivo, tumour tissues possess high concentrations of auxins and possibly other related compounds (Kulescha and Gautheret 1948, Link and Eggers 1941, and Locke, Riker and Duggar 1939). Tissue cultures of fully transformed crown gall tissues are not usually stimulated when grown on media containing auxin, and their growth is frequently inhibited (de Ropp 1947a, Hildebrandt and Riker 1947). This increased auxin content is either the result of decreased auxin destruction in crown gall tissues, or of increased synthesis. Conflicting results have been reported for the presence of IAA-oxidase in the tissues (Klein 1965) and it has not been resolved how the increases in auxin are obtained.

Gibberellins have been included in culture media to determine their effects on the growth of crown gall tissues. Growth was stimulated in Nicotiana tabacum and Melilotus officinalis crown gall tissues when gibberellin was added to the medium, while the

growth of Helianthus annuus, Vinca rosea, and Opuntia crown gall tissues was inhibited under exactly the same conditions (Nickell 1958).

Kinetin has been shown to inhibit gall formation when applied to leaves of Kalanchoe daigremontiana within three days of inoculation. When the leaves were wounded a few days before inoculation, kinetin did not have this inhibitory action on growth. Since the bacterial growth was only slightly decreased by the presence of kinetin, it was concluded that this substance acts on the plant (Brucker and Cziharz 1961). The reactions of crown gall tissues to kinetin alone have not been tested in a similar way to those of tissues grown on media containing gibberellin. However, as with auxin, it has been shown that crown gall tissues are a good source of cell division factors (Braun and Naf 1954, Steward, Caplin and Shantz 1955, Braun 1957a).

Braun (1956, 1957a, b) studied the growth characteristics and nutrient requirements of crown gall tissues in which the induction period had been stopped after varying lengths of time. When tumour cells are not fully transformed they do not grow as rapidly as cells which have undergone complete transformation and Braun postulated that the growth of such tumours might be limited by the ability of the cells to synthesize adequate amounts of all the required growth substances. He showed that as more slowly-growing tissues were used, representing a lower grade of transformation, more substances were required in the culture medium to increase tissue growth to that of fully autonomous tissues. Moderately fast-growing tumour cells required an auxin, glutamine

and meso-inositol as growth supplements, while a very slowly growing tissue required in addition to these, asparagine, cytidylic and guanylic acids. Braun has therefore suggested that several quite distinct systems synthesizing growth factors are permanently activated as a result of tumour induction.

Carrying this work further, Klein (1957, 1958) found that the supplements required for optimal growth were different for slow-growing tumours resulting from incomplete conditioning and slow-growing tumours formed as a result of incomplete induction or promotion. However, the suitability of the tissues Klein used for verifying the requirements of the different tumours was questioned by Braun (1962). Carrot phloem tissue was used in which normal and crown gall tissues were intermixed. Because of this, it was impossible to determine whether the supplementary substances in the medium were acting only on gall tissue or were stimulating the normal tissue.

From this review of crown gall it can be seen that although a great deal is known about the disease, the initial action of the bacteria on the plant tissues has yet to be elucidated.

b) Other Baterial Agents. Apart from crown gall there have been only a few reports of bacteria stimulating unorganized growth. Fallot (1958) reported that stem pieces of Vitis sp. in a dormant condition, grown in sterile culture, were stimulated to form callus when inoculated with Bacillus megaterium. Since that time this phenomenon has been studied intensively and in 1964 Fallot published his conclusions on the mode of action of this bacterium.

When B. megaterium was applied to dormant tissues of the vine 'Rupestris du Lot', proliferation occurred mainly in the region where the bacteria had been placed. With tissues taken from vines grown in the summer, the bacteria inhibited growth. However these tissues were induced to proliferate either in the presence of IAA, or with B. megaterium, when tissues exterior to the cambium were removed. When bacteria were applied to cultured tissue pieces of the vine variety, 'Syrah', which normally proliferates in winter, no stimulation of callusing resulted. B. megaterium was also responsible for the initiation of roots on numbers of explants both in the summer and the winter. The bacteria were also found to stimulate callus production in cultured tissue pieces of the tuber of Jerusalem artichoke, Helianthus tuberosus, although only a few roots were formed.

Fallot compared the action of B. megaterium on pieces of Jerusalem artichoke when applied immediately, eight and thirty days after the isolation of the tissue pieces; wounding was not required for the initiation of proliferation. In all cases the tissues proliferated, showing that the action of B. megaterium was quite different from that of A. tumefaciens. With the latter bacterium a definite conditioning phase associated with wounding is necessary before crown galls will develop. The action of B. megaterium was further shown to differ from that of A. tumefaciens when it was found that 60% of the explants, in which the bacteria were separated from the tissues by a collodion membrane, proliferated and formed roots. An investigation of the properties of the culture

filtrate was made. Two fractions were separated from ether extracts of the filtrate, the 'acid' fraction being active in callus formation and rooting, while the 'alkaline' fraction was active only in callusing. Analysis of the acid fraction showed that indole compounds and active compounds showing the characteristics of purine derivatives were present. The indole compounds were active in tissues not normally influenced by IAA. The type of growth induced by these compounds was quite different from that induced by IAA, showing that the active substances were different from known auxins. One of the purine derivatives was similar to a kinin in that it induced cell division in tobacco pith tissue when applied in the presence of IAA, but the other was inactive in this test.

In addition to B. megaterium Fallot (1960) found that Azotobacter chroococcum, Beijerinckia indica and Sarcina lutea also induced proliferation of Jerusalem artichoke tissue.

Other reports of bacterial stimulation of growth include those of Philipson and Sheat (1963) and Ark and Hunt (1966). In the former it was reported that Escherichia coli, Pseudomonas aeruginosa, Sarcina lutea and Aerobacter aerogenes stimulated the growth of decapitated sunflower hypocotyls. Ark and Hunt found that E. coli, E. intermedia and E. mutabilis all induced gall formation when injected into Datura plants. The only other known bacteria which might be thought to induce relatively undifferentiated galls are the species of Rhizobium found in the root nodules of leguminous plants. Allen (1954) has referred to them

as histologically reorganized root tissues, and because of their specialized function, they will not be considered in the present work.

1.4 Viral Agents

A number of viruses cause callus formation in infected plants in addition to the more usual symptoms of infection, but the wound tumour disease is one of the few well-documented examples of callus or tumour formation being the major symptom of infection with a virus. Black (1945) first reported the disease on sweet clover, (*Melilotus alba* and *M. officinalis*) and found that the virus which he called *Aureogenus magnivena* was carried by the leaf hoppers *Agallia constricta*, *A. quadripunctata*, and *Agalliopsis novella*. He also observed that formation of the tumours was associated with wounding. In roots, tumours usually develop near the wounds made by emerging lateral roots, while in stems where they occur less frequently the tumours form at points of stress or accidental wounds (Teitelbaum and Black 1954). Although the virus is systemic, a high proportion of it is concentrated in the tumours (Brakke, Vatter and Black 1954).

In addition to the requirement of wounding, it has been found that the genetic constitution of the plant influences the size and distribution of the tumours (Black 1965). The age of the plant also affects tumour formation, so that more tumours develop from younger tissues when wounded, than from more mature tissues (Black 1946). The formation of the virus wound tumours as well as their continued growth are temperature dependent (Selsky 1960).

At temperatures above 40°C or below 14°C fewer cuttings from infected sweet clover plants contain the virus.

Black and Lee (1957) found that when synthetic auxins were applied to plants infected with wound tumour virus, the number and size of stem tumours was greatly increased. Most stimulation came from applications of naphthalene acetic acid (NAA) which is not a natural auxin. With all auxins, roots were formed in addition to the tumours. These results support the possibility that some wound hormone is involved in the actual initiation of the tumours although it must be of a different nature from the known auxins (Black 1965).

The effect of NAA in stimulating the formation of wound tumours is closely comparable to the stimulation by auxins of crown galls induced by attenuated strains of bacteria (Braun and Laskaris 1942). In another respect, wound tumour is similar to crown gall in that it is capable of indefinite growth when small pieces are grafted to healthy plants (Black 1946). The virus is still present in the tissues however, and when the characteristics of this tissue are studied in tissue culture it is evident that although similar in some respects to crown gall, tissues of virus wound tumour are different in many ways from the bacterial-induced disease.

Although wound tumour tissue can be grown on artificial media in the absence of growth substances, the virus is still present so that the tissues cannot be regarded as fully autonomous in the manner of bacteria-free crown gall tissue. Sorrel wound tumour tissue has been grown in culture for many years but during this

time the character of both the tissue and the virus has changed and it would appear that both are closely correlated so that changes in one affect the other (Black 1957). The characteristics of crown gall tissues cultured over a number of years have remained unchanged. The wound tumour tissues differ from those of crown gall in a further respect. Low concentrations of IAA, NAA and 2,4-dichlorophenoxy acetic acid (2,4-D) stimulate growth in the virus-induced tissues (Nickell 1955) while auxins are usually without effect, or are inhibitory to growth of bacteria-free crown gall tissues (de Ropp 1947a, Hildebrandt and Riker 1947).

The growth requirements of wound tumour tissue have been studied in detail by Burkholder and Nickell (1949). They found that the tissues required phosphate in concentrations 10 to 100 times greater than for normal tissues, while increased nitrate also gave better growth. Other inorganic salts, sucrose, and B-vitamins were at normal levels. Nickell (1955) verified that this high phosphate requirement is associated with the tumour tissue and is not a growth requirement of normal clover tissues from which the tumours were formed. Normal tissues remained undifferentiated in comparison with tumour tissues which formed roots, particularly when grown in the dark.

In addition to the large phosphate requirement wound tumour tissues are unusual in another respect. They have been found to grow well on different carbon sources such as sucrose, glucose, fructose and raffinose, but in addition, good growth is maintained

when soluble starch is the only carbon source (Nickell and Burkholder 1950). Usually other tissues are unable to use starch in this way (Hildebrandt and Riker 1949, 1950, 1953). On investigation it was found that the wound tumour tissues contain an extra-cellular enzyme, α- amylase, which is not present in normal tissues of the species (Brakke and Nickell 1955).

Because of the presence of the virus in wound tumour tissues, certain nucleic acid and organic constituents have been found to stimulate growth through their action on the virus. Nickell, Greenfield and Burkholder (1950) showed that DNA inhibited growth but small amounts of alkaline-hydrolysed RNA were stimulatory. Components of the nucleic acids were tested for their effects on the tissues, when it was found that thiamine was without effect, uracil was stimulatory and adenine, very toxic. This was interpreted to mean that while adenine was inhibitory in both DNA and RNA, its effects in RNA were overcome by uracil. This inhibition of growth by adenine is in direct contrast to its promotion of leaf and bud growth in stem segments and callus of tobacco (Skoog and Tsui 1951).

Although the means by which the virus particles stimulate growth of wound tumours is not known, wounding is definitely implicated in the process leading to cellular transformation. The altered cells possess highly specialized properties which are not found in other undifferentiated forms of abnormal growth. These properties are due in part to the continued presence of the virus in the tissues.

1.5 Fungal Agents.

Although galls are formed on a number of plants as a result of infection with certain fungi, there has been little study of the physiological processess leading to gall formation. Most of the work has been concerned with determining the metabolic products formed by such fungi in culture. From this, deductions have been made on the way in which fungi cause the formation of galls.

In maize smut, a disease caused by Ustilago zeae, large galls are formed on various parts of the infected plant, and these galls have considerably higher levels of auxin than the normal plant tissues (Moulton 1942). U. nigra, like most smuts, does not induce gall formation in its host. When the two fungi were grown on a medium containing tryptophane, it was found that U. zeae produced IAA in appreciable quantities but there was no evidence of IAA in cultures of U. nigra (Wolf 1952). Moulton (1942) found that the pathogenicity of four strains of U. zeae could be correlated with the amount of auxin each produced in culture and Wolf (1952) suggested that this production of IAA was an important factor, but not the only one, in the development of galls in corn smut.

In similar work with Nectria galligena, the fungal pathogen causing canker disease of apple trees, Berducou (1952) showed that the fungus produced IAA when grown in a synthetic medium. A related species N. cinnabarina, which does not cause gall formation, did not produce IAA when grown under the same conditions. Again, the ability to form galls was correlated with production of IAA by the fungus in culture.

Culture filtrates of Aspergillus niger have been tested for their activity on bean plants. This fungus is not a known plant pathogen. It was found that application of the culture filtrate to the stems and leaf petioles caused malformations. Indirect evidence suggested that the active compound in this case was not IAA (Postlethwait and Curtis 1959).

The mycorrhizal fungi as a group represent a special case of invasion of plant roots with the subsequent malformation of root tissues. A symbiotic relationship exists between the fungus and the plant. Ulrich (1960) tested a number of mycorrhizal fungi for their ability to form IAA in culture and found that those which caused gall formation ('Knollen mykorrhizen') produced detectable amounts of IAA. Moser (1959) showed that mycorrhizal fungi were able to form indole compounds when grown in a culture medium containing tryptophane. More of these fungi were able to form indole compounds than fungi from other groups such as saprophytes from wood and straw.

The clubroot disease of crucifers caused by Plasmodiophora brassicae, is characterized by a swelling in the roots, in which abnormal cell growth and division occur. It is apparent that some stimulus from the parasite causes enlargement of cells in advance of those infected. Growth abnormalities are not as great in uninfected cells as in those containing the slime mould. This stimulus may be of a hormonal nature but it has been suggested that the cell division may be caused by mechanical pressures exerted by the adjacent dividing infected cells (Colhoun 1958). As

with the other fungal galls there is no evidence that the cells in clubroot become permanently altered so that they are autonomous in their growth requirements (Braun 1959).

Brian (1955) has stated that the growth substances formed by the fungi in culture need not necessarily be produced by them in the host plant, and gall formation may be induced in quite a different way. Auxins have be implicated in other forms of abnormal growth but in no case has it been shown that they are the sole causal agent. It therefore seems unlikely that this should be the only mechanism by which fungal galls are induced.

1.6 Insect and Nematode Agents.

A large number of insects classified in widely differing groups are able to induce gall formation in plants, but the continued growth of the gall is dependent on the presence of the insect (Maresquelle and Meyer 1965). In most cases the nature of the gall is dependent on the plant species together with the invading insect, and a highly specific structure is formed. Factors responsible for the initiation and continued stimulation of insect galls are not the same in all cases. This is not surprising when one considers the different methods of feeding of the insects. Some for example, remain on the outside of the plant and feed from the surface while others lie within the tissues (Mani 1964). The initial action of the insect is in wounding the plant tissues which prepares them for subsequent influences (Maresquelle and Meyer 1965). Following wounding, growth is stimulated by the secretion of some substances by the insect. Usually the substance originates from the salivary

glands of those insects which possess stylets; it is not known where the stimulus originates in insects with mandibles. Although there is a wide range of structure in insect galls, only those showing relatively undifferentiated tissues will be considered.

Whereas insect stimulation of plant growth usually occurs in conjunction with feeding there are several examples in which the stimulus originates from a different source. In the sawfly (Pontania sp.) which causes gall formation on willow leaves, cell division is induced at oviposition when the insect secretes a fluid from an accessory gland. It is thought that compounds with auxin activity are present in this secretion (McCalla, Genthe and Hovanitz 1962). A few cases have been reported of insect excrement causing cell division; Kuster (1911) showed that outgrowths formed near faeces of Pontania salicis and P. pomum. In contrast, Beck (1953) noted that in Solidago galls induced by larvae of the moth Gnorrimoschema gallaesolidaginis, cambial activity was partly inhibited at the base of the gall where the faecal matter was deposited. He found that the fine silky threads secreted by the larva over the surface of the larval chamber contained growth-promoting substances.

Attempts to define the nature of the stimulating substance have not produced any conclusive results. Extracts prepared from whole insects and tested at various concentrations, as well as extracts from salivary glands, have in some cases induced growth similar to that caused by the particular insect under consideration, although no large galls have been induced by this means (Maresquelle and

Meyer 1965). An active substance from larvae of Mikiola fagi has been shown to diffuse through lanolin and promote gall formation on beech leaves, but the tissues formed did not show the same characteristics as those induced by the larvae feeding directly on the leaves (Boysen Jensen 1948). Auxin has been shown to occur in a number of insect galls, particularly those caused by aphids (Link, Eggers and Moulton 1940, Nysterakis 1946, 1948). Indole compounds were found in extracts from nematode-induced root galls on Abelmoschus esculentus by Balasubramanian and Rangaswami (1962), while tryptophane was found in galls caused by the nematode Ditylenchus dipsaci in lucerne plants (Krusberg 1960). Egg sacs and larvae of the nematode Meloidogyne incognita were found by Sandstedt and Schuster (1963) to stimulate growth of carrot disks, and they concluded that growth-promoting substances must have been secreted by the nematodes. Thus, most information on the active substance points to the action of an auxin. It may be that the hyperauxinic nature of the tissues is caused by an insect secretion which is quite different from auxin, but which stimulates auxin production in plants. The insect secretion is more likely to be a variety of substances of a complex hormonal nature than a specific auxin (Maresquelle and Meyer 1965).

There is some evidence that the stimulating substance may not be an auxin. Anders (1956-1958), working on the aphid Phylloxera of vine reported that tissues were stimulated by combinations of amino acids, in particular tryptophane, histidine and glutamic acid. Other workers have found enzymes present in the

salivary glands which they consider to be active in gall formation. However, these substances were active only in isolated cases, and Maresquelle and Meyer (1965) do not consider the result to be of wide application.

Besides the study of stimulating substances produced by the insects and their action on plant tissues, the nature of some gall tissues and their growth requirements have been observed. Few isolations of insect gall tissue were successful until Pelet, Hildebrandt, Riker and Skoog (1960) isolated tissues from a number of insect gall types. They found that the general requirements for optimum growth were similar for normal tissues and for leaf galls on Vitis riparia caused by Phylloxera vastatrix. The endogenous level of the auxin type of growth substances seemed higher in gall tissues than in normal tissues although both of these tissues were dependent on the presence of growth factors for their continued growth. This behaviour is in sharp contrast to most of the abnormal growth forms already described which, in tissue culture, do not require added auxin.

There appears to be a variety of ways in which tissues from insect galls are stimulated. In addition to their direct action on plants, insects are also carriers of certain fungi, bacteria and viruses which can cause gall formation. The wound tumour virus which has already been described, is transmitted in this way.

1.7 Genetic Agents.

It is well established that the tumours arising 'spontaneously' on hybrids between certain species of Nicotiana, namely between the

glauca and langsdorffii groups, are of genetic origin, since inoculation experiments have failed to transmit the causal factor to other plants, and no bacterial or fungal parasite has been isolated from the tumours (Whitaker 1934). Although galls are formed as the result of genetic factors in other plants, only those occurring on tobacco hybrids have been studied intensively from a physiological aspect (Kehr 1965). A gall on spruce trees has been described in some detail (White 1958), but the causal agent has not been determined and the growth cannot be ascribed positively to a genetic disturbance. However, the spruce gall will be considered in the latter part of this section.

a) Nicotiana Hybrid Tumours. The hybrid tumours of Nicotiana form on the roots of seedlings but usually do not appear on the stems until the beginning of the flowering period when vegetative activity of the terminal meristem ceases (Whitaker 1934). The tumours which range in form from fasciations to undifferentiated growths (Levine 1937), are formed only when conditions are favourable for normal growth of the plant. They apparently form as the result of a disturbance in the growth-regulating mechanism which changes the normal pattern of growth, resulting in abnormal growth (Kehr and Smith 1954).

Brieger and Forster (1942) noted that tumour initiation occurred in necrotic cells and seemed to be associated with wound reactions. In plants subjected to radiation, tumours were formed more quickly than in untreated plants, because of physiological disturbances and damage to the tissues (Sparrow, Gunckel, Schairer, and

Hagen 1956). Braun and Stonier (1958) further emphasized the relationship between tumour formation and wounding. In direct contrast to this, Kehr and Smith (1954) and Kehr (1965) stated that no known stimulation from external agents was essential for the initiation of genetic tumours. Whitaker (1934) found that wounding the stem between nodes did not induce tumour formation and Izard (1952) showed that application of different concentrations of IAA, NAA, and 2,4-D did not appreciably modify the tumorous process. There thus appears to be evidence both for and against the necessity of wounding for tumour initiation. Hagen (1965) concluded that the formation of tumours can be increased by the application of some stress agent in the form of either chemicals, ionizing radiation, or wounding. With these agents, he suggested that metabolites collected at a wound or stress point, and this was the factor necessary for tumour induction.

Like virus wound tumour and crown gall tissues, genetic tumours have the property of continuing growth when grafted into healthy plants of related species which do not normally produce spontaneous tumours (White 1944).

The properties and growth requirements of genetic tumour tissues have been studied by growth <u>in vitro</u>. Skoog (1944) grew the tissues satisfactorily on media without added growth substances but this growth was not completely autonomous since it was stimulated by the addition of 0.2 ppm of IAA to the medium. He found that auxins were produced by tumour tissue cultures both in the light and the dark, and Kehr and Smith (1954) reported that hybrid tissues contained higher

levels of auxin than either of the parent tissues. More recent work by Bayer and Hagen (1964) showed that the effective auxin level was higher in the parental than in the hybrid tissues. This was so because although the hybrid tissues had higher concentrations of auxin, the levels of auxin inhibitors were higher than in the parental tissues so that the effective auxin level was lower. The growth requirements of hybrid tissues of an exceptional cross between N. glauca and N. langsdorffii, which do not form tumours, have been compared with those of the corresponding tumour-forming tissues by Schaeffer and Smith (1963). They found that the non-tumorous hybrid required both auxin and kinetin for rapid growth. The tumour tissues responded only slightly to these two substances. From this they concluded that the tumour tissues synthesized sufficient auxins and kinins for rapid growth, and that there was a close relationship between basic physiological processes in genetic tumours and crown galls. Further work with kinetin, glutamine and inositol showed that the tumorous cells resembled crown gall cells which had not been fully transformed (Schaeffer, Smith and Perkus 1963).

The tissues of genetic calluses show varying degrees of differentiation, and this was found by Skoog (1944) to depend on the composition of the medium supporting growth. On agar media, the tissues remained undifferentiated, but on liquid media leafy buds and stems were formed. The differentiation of tissues on the liquid media has been association with changes in oxygen gradients. The character of this tissue apparently changed with time since Braun and Stonier (1958) could no longer obtain differentiated tissues in liquid

culture after 300 passages.

More recently, Hagen (1965) concluded that the degree of organization of the tumour tissues was probably a function of growth substances such as IAA and kinetin, as well as the nitrogen supply. When both parent tissues were grown on a medium containing all of the substances necessary for good growth and differentiation of the hybrid tissues, those from the langsdorffii parent showed excessive leaf production and only a small amount of growth, while those from the glauca parent showed only slight internal differentiation and a large amount of growth. When hybrid tissues containing varying proportions of each parental genome were grown, differentiation in these led Hagen to conclude that growth and leaf formation appeared to be a function of the ratio of the two parental genomes.

As with the other forms of abnormal growth studied, the actual means by which hybrid tumours are initated are unknown, although it is evident that cellular alteration similar to that in crown gall occurs.

b) Spruce Tumour. White (1958) suggested that the tumour occurring on spruce is initiated by a somatic mutation in a single cell in the procambium of the bud, although he also considers it possible that a short-lived infection restricted to the bud stages might cause the cellular change. This could be a virus carried by the spruce bud aphid. Because of the difficulty in recognizing the galls in the first four to five years of growth however, no external inciting agents have been isolated and from the nature of the

abnormal growth, the former interpretation seems to be the most acceptable.

The tumours, which are found all over the tree but are most common near the ground, do not appear to affect the growth of adjacent normal tissues (Reinert and White 1956). In contrast to the abnormal growth forms described previously, the cells are regular and organized because the tumours arise as the result of a longer growing period of the vascular cambium than that giving rise to adjacent normal tissues. It is possible that the cambial initials differ physiologically from normal cells in their ability to grow well at lower temperatures (White 1958).

Growth requirements of normal and tumour tissues have been studied in an attempt to determine the physiological changes which have occurred to make the gall tissues different from neighbouring normal cells. Reinert and White (1956) found growth of tumour tissues to be much more erratic than that of normal tissues although the tumorous tissues formed callus two to three days before there was any trace of growth of normal tissues. These workers used a complex medium which contained tyrosine and tissues grown on this medium remained healthy for about two months before becoming brown. However de Torok and Thimann (1961) found that growth was greatly improved when tyrosine was omitted from the medium, and that when the other amino acids were excluded there was no decrease in the growth rate. With the original culture of both normal and tumorous tissues Reinert and White (1956) found that auxin was required and Reinert and Schraudolf (1959) reported that although normal spruce

tissue grew without auxin, a 50% increase in growth was obtained when IAA was added in optimum concentrations. De Torok and Thimann (1961) found no such increase in growth. Their work showed that the tumorous tissues required auxin for continued growth, while normal tissues did not require any auxin. When an antiauxin in the same concentration as the auxin was included in the medium it almost completely inhibited growth of the tumorous tissues, while normal tissues were unaffected. Growth resumed in the tumorous tissues as soon as the antiauxin was removed.

It is apparent that tissues from the spruce tumour differ markedly from most other forms of abnormal growth in their inability to grow in culture without auxin. The factor inciting abnormal growth in this case apparently acts on quite a different growth system from those previously studied.

1.8 Conclusions on the different forms of abnormal growth. The aim of the present experimental work.

In most of the forms of unorganized abnormal growth which have been described, wounding must occur before abnormal growth is initiated by the particular inciting agent. In crown gall formation, wounding has been shown to condition the cells during a well-defined period of time before the bacteria can induce tumour formation. In the other growth forms however, no such clearly delimited function of wounding has been demonstrated. With wound tumour disease, the virus may be present in the tissues for some time without any evidence of tumour formation. Tumours are only initiated when some tissues are wounded. Similarly with the Nicotiana hybrids, the

potential of the cells to become transformed is always present but normal growth occurs until some stress or wound initiates tumour formation.

The growth substance metabolism becomes changed in most forms of abnormal growth so that the tissues are less dependent on auxins and cytokinins than are corresponding normal tissues. The greatest changes of this form occur in crown gall tissues. Virus and genetic tumours, and habituated tissues have no absolute requirement for auxin but their growth may be increased by it. In addition these tissues show greater degrees of dependence on other growth supplements. Tissues from the tumours formed on spruce, in direct contrast to those just described, require auxin for their continued growth.

It is apparent that wounding in some way places cells in a state where some basic metabolic system which influences growth substance metabolism can be changed by the inciting agent. This change may be permanent as in the case of crown gall tissues or it may be only temporary. For example, crown gall tissues continue their abnormal growth after removal of the bacteria, while in wound tumours abnormal growth continues only in the presence of the virus. The metabolic process in the spruce galls appears to be affected in a different way so that less instead of more auxin is produced. Whatever the system affected, it appears to be altered to varying degrees in the different growth forms. It is likely that the same metabolic system is influenced in most cases because of the similarity of the different tissue requirements.

In contrast to those growth forms in which wounding is a pre-requisite for abnormal growth, the action of Bacillus megaterium on vine tissues is caused only by metabolic products of the bacteria. In this case, wounding is without influence on their effects.

The aim of the following experimental work was to examine the effects of the bacterium, Escherichia coli, on plant tissues. Its stimulating action on sunflower hypocotyl tissue was reported by Philipson and Sheat (1963). Agrobacterium tumefaciens induces profound changes in plant cells so that autonomous growth results. The only other bacterial-induced plant proliferation which has been described in detail is that caused by Bacillus megaterium and this is of quite a different nature from crown gall. It was of interest to determine in what way E. coli acts on plant tissues and whether it conforms with either the reported characteristics of A. tumefaciens or B. megaterium, or with any known agent inducing abnormal growth.

C H A P T E R T W O

MATERIALS AND METHODS

2.1 Selection of Test Plant.

When studying pathogen-host plant relations, the infected plant or tissue is often grown under sterile conditions, so that the effects of the pathogen alone can be observed. This excludes possible complications from secondary contaminants.

In order to study the effects of certain bacteria which are not normally plant pathogens, in particular Escherichia coli, a plant suited to growth under aseptic conditions was required. The sunflower, Helianthus annuus var. Giant Russian, was found to be suitable and was used throughout the experimental work. The seed of the sunflower is exalbuminous and in this variety, measures 1.5 x 0.8 cm. The outer seed coat is hard and was found to act as a good shield against invasion of the embryo by the many micro-organisms present on the surface of the seed. This coat could be removed easily and in its absence, seeds germinated at a more uniform rate than with the coat, producing a young plant with a hypocotyl 3 to 4 cm long in four days, when grown in the dark. Growth was somewhat slower in the light.

Kupila (1958) has shown that the tissues of sunflower are cytologically stable compared with those of tomato and pea, both of which show a varying degree of polyploidy. Helianthus annuus plants or tissue cultures have been frequently used as the plant

TABLE 2.1 WHITE'S MODIFIED MEDIUM

		mgm/litre	Stock solution concentrations relative to the final medium
I	$Ca(NO_3)_2 \cdot 4H_2O$	288	x 1,000
	KNO_3	80	
II	KCl	65	x 1,000
	$NaH_2PO_4 \cdot 2H_2O$	21.5	
III	$MnCl_2 \cdot 4H_2O$	6	x 1,000
	$ZnSO_4 \cdot 7H_2O$	2.65	
	KI	0.75	
IV	Na_2SO_4	200	x 100
	$MgSO_4 \cdot 7H_2O$	740	
	H_3BO_3	1.5	
V	H_2MoO_4	0.0017	x 2,000
VI	$CuSO_4 \cdot 5H_2O$	0.02	x 400
VII	$FeCl_3$	4.8	x 100
	2Na EDTA	8	

host in the study of one pathogenic bacterium in particular, Agrobacterium tumefaciens. Culture of both normal tissue and the tumour tissues of crown gall have been of importance, since the release of the tissue from the influences of adjacent cells in the whole plant has provided a valuable means of observing fundamental physiological differences between the two types of tissue.

2.2 Growth Media.

a) Preparation and Composition of Tissue Media. Sterile media were needed to support the germination and growth of sunflower seedlings, and the growth of segments of the hypocotyl. De Ropp's medium (1951c) was found to be suitable for both these functions. This consists of 2% sucrose, White's mineral solution and 1% agar. White's medium as modified by Boll and Street (1951) was used, and the composition of this medium is shown in Table 2.1. The concentrations of some of the salts differ from the original White's medium, but these have been found satisfactory over some years of use as a tissue culture medium. Solutions I to VI were combined in a stock solution at a concentration of 10 x standard and the iron solution (VII) was added when the complete nutrient with sucrose and agar had been prepared. All solutions were prepared and diluted with pyrex distilled water. Stock solutions were stored in dark bottles at $4^{o}C$ and the 10 x standard mineral solution was stored in a pyrex flask at this temperature.

Initially, ferric chloride was used alone as the source of iron but a flocculent precipitate sometimes formed in the medium after autoclaving. Addition of the chelating agent ethylene-

diamine-tetra-acetic acid (EDTA) prevented the formation of this precipitate and its use was continued.

The complete de Ropp's medium was prepared immediately before use. Agar in approximately the right proportion was added to either 25 x 150 mm pyrex tubes, or 100 ml Erlenmeyer pyrex flasks, and the solution was then dispensed to the containers, 25 or 50 ml respectively. The containers were stoppered with gauze-covered cotton-wool bungs, and were covered either with non-absorbent cellophane in the case of tubes, or with 50 ml pyrex beakers, in the case of the flasks. These were then sterilized in a steam autoclave for 20 minutes at 120°C. As soon as the tubes and flasks were cool enough to handle, each was rotated to distribute the melted agar evenly through the medium. The final pH of the medium was 5.5.

All the glassware used throughout the experimental work was thoroughly cleaned by washing in hot water and detergent, after which it was coated with a dichromate-sulphuric acid mixture and left for at least 12 hours. Finally, all pieces were carefully rinsed to remove all traces of the acid.

When indole acetic acid (IAA) was required in the medium it was added before autoclaving from a stock solution containing 50 ppm, stored in dark glass at 4°C. Gautheret (1959) found IAA to be stable at 110°C and in the present study the amount of decomposition through autoclaving was considered to be negligible. When light of normal wave-length is present no decomposition occurs unless certain photo-dynamic compounds such as riboflavin, methylene blue, or eosin are present. In ultra violet light, breakdown ofIAA also occurs,

TABLE 2.2 SKOOG'S MEDIUM

		mgm/litre	Stock solution concentrations relative to final solution
I	NH_4NO_3	1650	Each salt as a separate solution at x 100
	KNO_3	1900	
	$CaCl_2$ (72% dried)	480	
	$MgSO_4 \cdot 7H_2O$	370	
	KH_2PO_4	170	
	FeNaEDTA	38.5	
II	H_3BO_3	6.2	Each salt as a separate solution at x 1,000
	$MnCl_2 \cdot 4H_2O$	19.8	
	$ZnSO_4 \cdot 4H_2O$	10.6	
	KI	0.83	
	H_2MoO_4	0.16	
	$CuSO_4 \cdot 5H_2O$	0.025	
	$CoCl_2 \cdot 6H_2O$	0.025	
III	Sucrose	30,000	
	Edamin (casein hydrolysate)	1,000	
	Myo-inositol	100	
IV	Glycine	12	
	Thiamine	0.4	
	Pyridoxine HCl	0.4	
	Niacin (nicotinic acid)	2.0	
V	Indole acetic acid (IAA)	2.0	
	Kinetin	0.5	

(Galston and Hillman 1961). However, although degradation in the light may take place it does not perceptibly diminish the growth-promoting properties of the auxin (Gautheret 1959). In spite of these disadvantages, IAA was used as a growth promoter because it occurs naturally in plants, whereas naphthalene acetic acid (NAA), which is more stable, does not. Further, when excess concentrations are used IAA does not immediately produce toxic effects.

In preliminary experiments small specimen tubes (15 x 50 mm) were placed in the larger tubes (25 x 150 mm) which were stoppered before autoclaving. The sterile medium was aseptically dispensed into the specimen tubes, 15 ml per tube. The specimen tubes were used inside the larger tubes so that the grown seedlings in the tubes could be lifted out, decapitated and inoculated, using the forceps and scalpels available at that time. Later modifications of technique made this procedure unnecessary.

While de Ropp's medium was suitable for the growth of both seeds and sections of the sunflower hypocotyl, another medium was required for the growth of pith tissue. The culture of pith tissue not containing adjacent cambial areas, requires extra growth substances and vitamins. The medium described by Murashige and Skoog (1962) supplied these factors and was used with some slight modifications to the vitamin constituents (Table 2.2). The organic constituents were added only when preparing the complete medium. All the vitamins were combined in a 1000 x standard solution which, together with kinetin, was stored in the frozen state. The pH of the prepared medium was adjusted to between 5.7 and 5.8 with 0.1 molar HCl. The

medium was dispensed to tubes, or flasks containing agar, and autoclaved in the same manner as for de Ropp's medium. When tubes of this medium were prepared they were sloped during setting, to provide a greater surface area on which the pith tissue could grow.

In both de Ropp's and Skoog's media, the inorganic salts and EDTA used were Analytical Reagent Grade. IAA, kinetin, the vitamins and the amino acids were obtained from L. Light and Company.

b) Preparation and Composition of Bacterial Media. Dehydrated Difco Bacto nutrient broth and agar were used initially for the culture of E. coli and A. tumefaciens. This medium contained 3 g of bacto-beef extract and 5 g of bacto-peptone per litre of solution. Throughout the experimental work, this medium was used for the culture of A. tumefaciens, but a synthetic medium was found which would support the growth of E. coli. The synthetic medium (Pelczar and Reid 1958) contained the following compounds (Analytical Reagent Grade):

	g/litre
$NH_4H_2PO_4$	1.0
NaCl	5.0
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4	1.0
Glucose	5.0

The strain of A. tumefaciens used throughout the experimental work was received from the Plant Diseases Division of the Department of Scientific and Industrial Research, while that of E. coli was obtained from the University of Otago Microbiology Department (no.149). Identification of this culture was confirmed by Miss L. Williams of

the Department of Microbiology, University of Queensland. A culture of Bacillus megaterium from the Microbiology Department, Massey University was also used in one experiment.

Twenty-four hour cultures of the bacteria were used throughout the work, except where otherwise stated, and were grown in the dark incubator at 25°C. Although this was not the optimum for bacterial growth, it was the temperature at which the plant tissues were grown.

2.3 Incubators and Growth Cabinets.

The plants were all grown at 25°C ± 2°, but in one of three different light intensities.

i) In the dark: plants were grown in an incubator not fitted with lights.

ii) Low light intensity: a small growth cabinet was used in which light was supplied by eight 40 watt "warm white" fluorescent tubes, with six 60 watt "Osram" strip tungsten filament lights, these latter balancing the light spectrum by increasing the red wave lengths. The light intensity was about 300 foot candles and the plants received twelve hours of light during each twenty-four hour period. This cabinet was subject to more temperature fluctuation than the other two places in which growth occurred but the cabinet was only used in one experiment.

iii) High light intensity: plants were grown in a growth room containing a bank of forty-eight "warm white" 80 watt fluorescent tubes supplemented by twenty-seven 15 watt tungsten lamps; this provided a light intensity of approximately 2,000 foot candles.

The plants received fourteen hours of light in each twenty-four hour period, and during darkness the temperature was 2 to 3°C lower than during the light period.

2.4 Plant Techniques.

a) Preparation and Treatment of Seeds. Bacterial cultures and plant material which was to remain uncontaminated were handled in an inoculating room sterilized by spraying with a 1% solution of thymol in alcohol twenty minutes before entering the room. The inoculating room remained reasonably sterile over the period it was in use (up to three hours), because there was a tight seal on the door and incoming air was pumped through a cotton-wool pad in the wall of the room.

Initially, sunflower seeds were surface sterilized in 20 volume hydrogen peroxide, after the removal of the outer seed coat. Without washing in water they were planted in sterile tubes with the radical end embedded in de Ropp's medium. They were germinated in either the dark incubator or the growth room. After some time it was apparent that peroxide was not effective enough as a disinfectant. A number of different sterilizing agents were therefore tested for their ability to destroy the surface microflora of the seeds. These included mercuric chloride, "Zephiran" (a mixed high molecular weight alkyl-dimethyl benzyl ammonium chloride), and "Janola" (containing 3 to 5% of available chlorine as sodium hypochlorite). The seeds were immersed for varying times and after treatment, were planted in de Ropp's medium to determine their sterility and the phyto-

TABLE 2.3 PHYTOTOXIC EFFECTS OF SURFACE STERILIZING AGENTS ON SEEDS, AND THEIR DISINFECTANT ABILITY.

TREATMENT	TIME OF IMMERSION	NUMBER INFECTED OUT OF 10 TREATED	COTYLEDONS DAMAGED	ROOTS DAMAGED	PLANT GROWTH
H_2O_2 25 volume	10 mins.	2	1	3	Uneven
	15 "	3		1	growth.
	20 "	3	6	6	
5% Zephiran	5 "	0	10	10	Seeds black-
	10 "	0	9	10	ened and
	15 "	0	9	10	little growth.
10% Janola	5 "	0		1	Healthy
	10 "	0	1	1	growth.
5% Zephiran &	5 & 10 "	0	8	10	Little growth
10% Janola	10 & 5 "	0	7	10	and blackened
	5 & 5 "	0	9	10	cotyledons
0.1% $HgCl_2$	5 "	0		10	Little growth
	10 "	0	2	9	although
	15	0	1	10	green.
Distilled water		10			

toxicity of the disinfectant used. The results of this test are shown in Table 2.3. A 10% aqueous solution of "Janola" for 5 to 15 minutes proved to be best since it allowed even germination of the seeds, while the roots and cotyledons sustained little damage as compared with the hydrogen peroxide treatment.

b) Preparation and Treatment of Whole Hypocotyls. For the preliminary experiments in which the effects of bacteria on whole hypocotyls were studied, the seeds were germinated and grown until the hypocotyls were 4 to 5 cm long. The cotyledons were detached 2 to 3 mm below the cotyledonary node, by means of a cut at right angles to the main axis. It was important that this cut was made horizontally so that each tissue of the stem was evenly exposed to the bacteria. Initially the only way this could be achieved was by growing the seedlings in specimen tubes inside test-tubes. The specimen tube was then withdrawn and the cotyledons removed, following which the cut surface was inoculated. A better method was developed, lessening the possibility of contamination during decapitation and inoculation, as well as providing a greater volume of nutrient medium for the subsequent growth of the decapitated seedling. An instrument was made which could be inserted into the tube and cut the cotyledons off cleanly. It consisted of a small piece of razor blade inserted in a holder on the end of one arm of a pair of forceps which closed on to a piece of "teflon" attached in a corresponding position on the other arm. This instrument was easily sterilized by flaming.

The cut surface was inoculated with a drop of bacterial culture suspended in a platinum loop. Standard microbiological techniques were observed in carrying out inoculations.

c) Preparation, Treatment and Harvesting of Hypocotyl Disks. Plants with hypocotyls 1.5 to 2.5 cm long were used for cutting into disks. Each plant was withdrawn from its tube of agar and placed on a filter paper which had been previously wrapped in cellophane and sterilized by autoclaving in a petri dish. The hypocotyl was decapitated 2 mm below the cotyledonary node and segments 1 mm thick were cut from below this. Except where otherwise stated these segments were placed in 100 ml flasks with the radical end in contact with the medium. The disks were set out in serial order, ten being the usual number cut from each hypocotyl. The prepared disks were inoculated with a loopful of the appropriate bacterial suspension. Initially, flasks were plugged by gauze-enclosed cotton-wool bungs, but it was found that the agar medium dried out over long periods, particularly when the flasks were left in the growth room. Squares of aluminium foil, sterilized by dry heat, were moulded to cover the tops of the flasks. These were found to be effective in allowing adequate passage of air, and at the same time reducing the dehydration of the medium.

This method of growing sunflower hypocotyl disks was described by de Ropp (1951c), who grew 5 mm sections of sunflower hypocotyl radical end uppermost, on the same medium.

At harvesting the hypocotyl disks were taken from the surface of the agar and surplus moisture was removed by rolling them on blotting paper. When roots were formed which grew through the medium, the agar was emptied from the flasks and the roots were dissected out and quickly dried on blotting paper. All the pieces of tissue from one flask were placed in a chromic acid-cleaned weighing bottle of known weight which had previously been dried at 100°C and cooled in a desiccator. The fresh weights of the hypocotyl disks and of tissues derived from them were thus determined. Tissues were dried at 100°C and cooled in a desiccator for 12 hours before being weighed. Initially, this process was repeated until constant weight was attained to within 0.0005 g. At a later stage in the experimental work, a balance of greater accuracy was obtained and the weights of the tissues were measured to within 0.0001 g.

d) Preparation of Pith Cultures. A line of sunflower pith tissue was established on Murashige and Skoog's medium, according to the standard methods outlined by Gautheret (1959). The growth of this tissue was maintained by transferring 2 to 3 mm cubes, cut on sterile filter paper, to tubes containing slopes of Skoog's medium. Healthy growth was obtained on this medium at 25°C.

CHAPTER THREE

PRELIMINARY EXPERIMENTAL WORK

Prior to the present investigation studies in this laboratory had shown that certain bacteria including Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa and Sarcina lutea were able to induce the formation of calluses in decapitated hypocotyls of sunflower seedlings (Helianthus annuus), comparable in size and character to those initiated by Agrobacterium tumefaciens (Philipson and Sheat 1963). It had been found that E. coli induced the formation of very large calluses more frequently than the other bacteria. However, the studies which determined this were purely qualitative and no measurements of the numbers of hypocotyls forming calluses were made.

Only two other reports of acceleration of growth by non-pathogenic bacteria had been made. Zielinski (1955) found that an uncharacterized bacterium stimulated the growth of carrot tissue cultures, while Fallot (1958) demonstrated increased cell division in cultures of Vitis rupestris in the presence of Bacillus megaterium. Since the commencement of the present investigation Ark and Hunt (1966) have reported that E. coli, E. intermedia and E. mutabilis suspended in human plasma and inoculated into stems and leaves of small Datura plants, caused the development of tumours on the stems and spongy cushions of tissue on the leaves which later

became firm rough nodules. Sunflower plants also responded to inoculation with these bacteria but the formation of galls was exceedingly slow. Members of the Enterobacteriaceae have also been isolated from brown bast of rubber trees (Hevea brasiliensis). This disease is characterized by considerable callus formation at branch forks (Taysum 1963 - private communication).

In the first stage of the experimental work the methods of Philipson and Sheat (1963) were used to test and record in detail the reactions of decapitated hypocotyls to inoculation with E. coli and A. tumefaciens.

EXPERIMENT 1. THE EXTENT OF THE RESPONSE OF HYPOCOTYLS TO INOCULATION

Bloch (1941) emphasized that in studying abnormal plant growths, in particular those with a simple undifferentiated structure, a thorough knowledge of the normal wound response of the tissues is necessary before interpretation of the abnormal structures can be made.

An experiment was therefore designed to determine the normal wound response of uninfected tissues at the cut surface of decapitated sunflower hypocotyls, and to provide details of the actual numbers of hypocotyls forming callus when inoculated with E. coli. Philipson and Sheat's note (1963) was descriptive and gave no indication of the extent of the response of the decapitated sunflower hypocotyls to infection with the various bacteria. The first experiment was therefore an attempt to define the problem

arising from the report of Philipson and Sheat.

3.1 Experimental Details.

Of 100 sunflower seeds which were sterilized and planted in tubes, contained in 25 x 150 mm test tubes, 60 were grown in the dark incubator and 40 in the growth room under high light intensity. Seedlings growing in the dark reached the required height (4 to 5 cm) in three to four days and were then decapitated and inoculated; 17 with a 48-hour culture of E. coli in nutrient broth, 16 with sterile nutrient broth, and 17 with sterile distilled water. Ten seedlings were discarded due to infection. The inoculated hypocotyls were returned to the dark incubator, and the resulting proliferation was recorded 22 days later. Seedlings grown in the light required a longer growing period than those in the dark to reach a similar size. Seven day old seedlings were decapitated, 16 being inoculated with E. coli cultures as before, and 17 with sterile nutrient broth. Seven seedlings were discarded owing to infection. Results were recorded after 18 days growth in high light intensity.

Oginsky and Umbreit (1955) state that serial transfers through a susceptible host can increase the virulence of strains of bacteria which have been cultivated for some time in the laboratory. This is a common bacteriological procedure. Philipson and Sheat (1963) reported a three-fold increase in the number of inoculated hypocotyls forming callus when the E. coli used was re-isolated from a callus. The strain of E. coli (ECS_4) used in

the present experiment, was selected for virulence by four such passages through sunflower tissue.

3.2 Results and Discussion.

The hypocotyls reacted to decapitation and inoculation by the formation of callus tissue at the cut surface, or by swelling either immediately at, or some distance below, the cut surface. Combinations of these characters also occurred. This was so whether the inoculum consisted of the bacterial suspension, the nutrient broth, or the sterile water. In a number of cases the hypocotyl died, or no reaction was shown in the tissues. The general type of response can be seen in Figures 3.2 and 3.9.

Of the hypocotyls grown in the dark only two showed a large amount of proliferation from the cut surface, which appeared to be more than the normal wound response. One of these was infected with E. coli, and the other treated with sterile broth. Hypocotyls inoculated with distilled water swelled.

Because a large variety of reactions occurred it was difficult to assess the normal wound response and the increased response caused by the presence of the bacteria. The hypocotyls were therefore recorded as showing a response if either callusing or swelling occurred (Table 3.1).

TABLE 3.1 RESPONSE OF HYPOCOTYLS TO INOCULATION

TREATMENT	NUMBER OF HYPOCOTYLS			
	POSITIVE RESPONSE	NO REACTION	DEAD	TOTAL
DARK				
E. coli	9	7	1	17
Distilled water	4	5	8	17
Nutrient broth	7	6	3	16
LIGHT				
E. coli	2	3	11	16
Nutrient broth	0	5	12	17

With hypocotyls grown in the dark, inoculation with *E. coli* did not significantly* increase the amount of callusing or swelling compared with those treated with nutrient broth. Hypocotyls inoculated with distilled water had a significantly higher death rate (at the 2.5% level in a χ^2 test, in Appendix I) than those inoculated with nutrient broth or *E. coli*. In the light, as in the dark, there was very little difference between the reactions of

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* When a result is obtained which is unlikely to have arisen by chance it is statistically significant (Moroney 1962). Thus when an event happens which is very improbable on the basis of simple sampling, the difference is said to be highly significant.

hypocotyls inoculated with E. coli and with sterile nutrient broth. Since the seedlings were grown in only 15 ml of medium, it is probable that their nutrient supply was quickly exhausted thus limiting the growth of tissues. This would explain why little difference was observed between the treatments.

The nutrient broth used contained 0.2% tryptophane and it is likely that this decomposed during autoclaving to give IAA, (Burkholder 1939, Roberts and Street 1955, Fallot 1964). The IAA would act as a stimulus to callus formation, and may have been responsible for the increased callusing observed in one of the broth-inoculated hypocotyls.

A piece was taken from each of the largest calluses on hypocotyls grown in the dark and inoculated with E. coli, sterile nutrient broth, and distilled water. These were sub-cultured to nutrient agar slopes and incubated at 25°C. Bacteria emerged only from the tissue taken from the callus inoculated with E. coli showing that the proliferation induced by inoculation with nutrient broth and distilled water was not the result of contamination by bacteria.

These results with inoculated hypocotyls indicated that greater numbers of seedlings and a more virulent strain of bacterium would have to be used in future experiments to obtain meaningful results.

The lack of virulence of the E. coli was of real concern. A further passage of the culture through sunflower was carried out so that a strain of increased virulence could be selected.

It was hoped that this selection would result in a higher percentage of infected hypocotyls forming callus, as reported by Philipson and Sheat (1963). On plating out the selected ECS_5 culture, contaminants differing in colony appearance were found to be present. The sunflower seeds used for the initial experimental work were of poor quality and in spite of surface sterilization, it was found that a number of seedlings showed signs of infection with fungi after a week's growth. By this time the plants had been inoculated with E. coli, and selection of a more virulent strain may have occurred from a contaminated plant.

The original culture of E. coli was used throughout subsequent experimental work, and the cultures which had been selected for increased virulence by plant passage were only used where noted.

EXPERIMENT 2. DETERMINATION OF THE NUMBER OF REPLICATES REQUIRED.

Because of the unsatisfactory results of the first experiment an attempt was made to determine the number of replicates of each treatment needed to give statistically significant differences between the treatments. If this were established, constant numbers of plants could be used in later experiments. By dissecting the larger calluses formed and subculturing from these it was also hoped to establish a clone of E. coli-induced callus free from the restraints of adjacent normal plant tissue.

The seedlings were grown in flasks containing 50 ml of nutrient medium in order to prolong their life. In the previous

experiment 15 ml had been used and this was now thought to be insufficient for the period of growth required.

3.3 Experimental Details.

Seeds were planted in 100 ml Erlenmeyer flasks containing 50 ml of de Ropp's nutrient agar, and were grown in the dark. After three to four days some of the seedlings were decapitated and inoculated with 48-hour or 24-hour cultures of E. coli (ECS_4) before it was discovered that this strain was contaminated. The remainder of the plants were inoculated with either the original strain of E. coli or with sterile nutrient broth which was used as a control. All hypocotyls were grown in the dark and the results were recorded after two to four weeks. Three of the E. coli-inoculated hypocotyls which had formed large calluses were subcultured after two and a half weeks into freshly prepared flasks containing 50 ml of de Ropp's medium.

3.4 Results and Discussion.

A wide range of responses occurred in the hypocotyls, similar to those described in the first experiment. The results are recorded in Table 3.2. The response of callusing by the cut surface was regarded as normal when only a little proliferation occurred, classed as + or ++ depending on the size. Wound responses greater than normal were classed as +++ or ++++, according to the size of the callus formed. Hypocotyls which swelled were classified as having proliferated a little, +.

TABLE 3.2 RESPONSE OF HYPOCOTYLS TO INOCULATION

TREATMENT	NUMBER OF HYPOCOTYLS						
	POSITIVE RESPONSE				NO REACTION	DEAD	TOTAL
	+	++	+++	++++			
E. coli (ECS_4)	5	8	7	5	2	14	41
E. coli	3	3	6	3	1	4	20
Nutrient broth	5	3	3	0	0	10	21

When the original strain of E. coli was compared with the strain selected for virulence (ECS_4), fewer calluses were formed on hypocotyls inoculated with ECS_4 although there was no significant difference between the numbers of hypocotyls showing more than the normal wound response. Similarly there was no significant difference in response between uninfected controls and hypocotyls inoculated with strain ECS_4. The controls formed fewer large calluses than the infected hypocotyls. There was, however, a difference, significant at the 10% level, between hypocotyls treated with the original strain of E. coli and sterile nutrient broth. The infected plants formed three times as many large calluses as the controls. (For details of significance tests, see Appendix I).

Using the number of large calluses formed as the criterion, calculations were made of the number of replicates required to show a difference between inoculated and uninoculated treatments, significant at the 1% level, 75% of the time (see Appendix I). The figures used in this calculation were obtained from hypocotyls inoculated with the original strain of E. coli, showing maximum

proliferation. No such proliferation occurred with the sterile nutrient broth treatment. The number of replicates was found to be 29.

The hypocotyls which were subcultured into flasks containing fresh medium showed only a little further growth before becoming necrotic. Consequently, the intention of dissecting the callus for continued growth of the tissue was abandoned.

EXPERIMENT 3. EFFECT OF HOLDING SEEDLINGS AT LOW TEMPERATURES ON CALLUS FORMATION.

Since the sunflower seeds did not germinate uniformly, it was thought that some of the seedlings would have to be stored at $4^{\circ}C$ until sufficient plants of the same size had accumulated to be inoculated at the same time. This would be necessary in view of the large number of replicates needed for each treatment.

An experiment was set up to determine whether the capacity of sunflower hypocotyls to form calluses in the presence of E. coli was affected by cold storage or by the age of the seedlings at the time of decapitation and inoculation.

3.5 Experimental Details.

Sterilized seeds were planted in 100 ml flasks containing 50 ml of de Ropp's agar, and were germinated and grown in the dark incubator. As the length of the hypocotyls reached 4 cm, half of the seedlings were stored at $4^{\circ}C$ and the other half were inoculated immediately with E. coli. After the seedlings reached the required size they were kept in cold storage for one, three, or seven days

TABLE 3.3 RESPONSE OF HYPOCOTYLS STORED AT 4°C FOR DIFFERENT PERIODS AND INOCULATED WITH E. COLI

ACTUAL AGE (DAYS)	DAYS AT 4°C	NUMBER OF HYPOCOTYLS						
		POSITIVE RESPONSE				NO REACTION	DEAD	TOTAL
		+	++	+++	++++			
3	0	1	1					2
4	0	2	3			1	6	12
	1						1	1
5	0	3		2	1	2	8	16
	1					1	3	4
6	0	1		2		1	4	8
	1	2	1	1			2	6
	3			1				1
7	0	1				1		2
	1	1					2	3
	3				1		3	4
8	0				1	1	1	3
	1	1						1
	3	3	1				2	6
9	1						1	1
	3					2	1	3
10	0	1					1	2
	3		1					1
	7		1					1
11	3		1					1
	7		1			1	2	4
12	7		3				3	6
13	3						1	1
	7	1				1	1	3
14	7	1				1		2
16	7						1	1
TOTAL		18	13	6	3	12	43	95

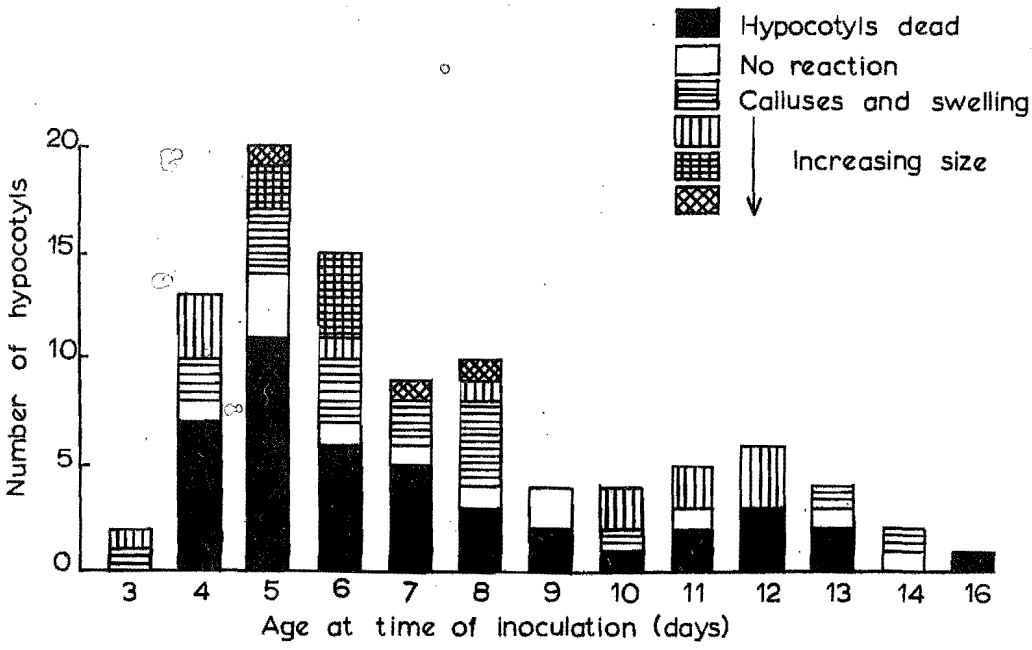


FIGURE 3.1a DISTRIBUTION OF REACTING HYPOCOTYLS WITH ACTUAL AGE

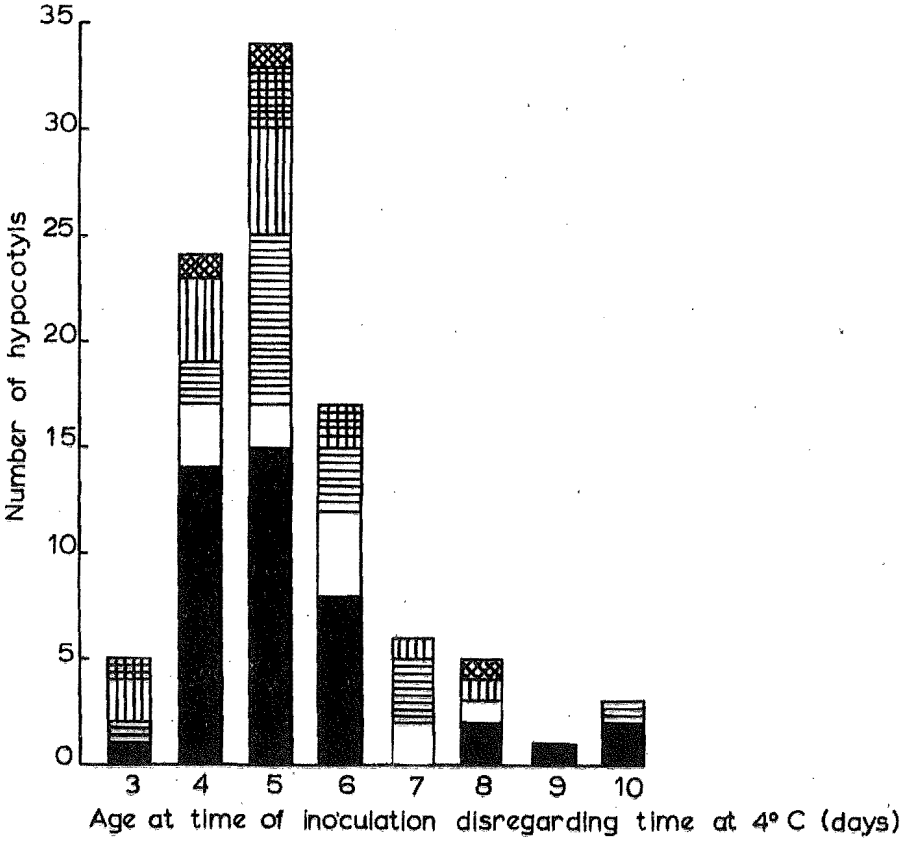


FIGURE 3.1b DISTRIBUTION OF REACTING HYPOCOTYLS WITH PHYSIOLOGICAL AGE

before being inoculated and replaced in the dark incubator. For every five hypocotyls inoculated with E. coli, one was inoculated with nutrient broth. Results were recorded 18 and 30 days after the beginning of the experiment.

3.6 Results and Discussion.

The reactions of the hypocotyls are recorded in Table 3.3. A better representation was gained by grouping the plants according to their actual age, rather than their physiological age i.e. their age when placed in the refrigerator. This is illustrated in Figure 3.1 a, b. Large calluses occurred in plants five to eight days old regardless of whether or not they had been stored at $4^{o}C$. Plants three, four, ten, eleven and twelve days old showed quite a marked callusing response while older plants showed little if any proliferation (Figure 3.1a). When plants were grouped according to their physiological age, this time being taken from the time they were planted to when they were stored, large proliferations were found in a much greater range of age groups.

It appeared that although growth in length of the seedlings was retarded by storage at low temperatures, some of the processes of aging still continued, so that callus formation was reduced in older plants.

When the age of the hypocotyls was disregarded, and the plants were grouped according to the length of cold treatment (Table 3.4), it was found that hypocotyls stored for one day had a higher death rate than those stored for longer. This result may have been

due to chance. The increased death rate was partly compensated for by a lowering of the number of hypocotyls showing no reaction.

TABLE 3.4 CALLUSING AND SWELLING IN INOCULATED HYPOCOTYLS HELD AT 4°C FOR VARYING TIMES BEFORE INOCULATION

DAYS AT 4°C	NUMBER OF HYPOCOTYLS						
	POSITIVE RESPONSE				NO REACTION	DEAD	TOTAL
	+	++	+++	++++			
0	9	4	4	2	6	20(44%)	45
1	4	1	1	0	1	9(56%)	16
3	3	3	1	1	2	7(41%)	17
7	2	5	0	0	3	7(41%)	17

Plants which had been stored for three days before inoculation showed a higher proportion of calluses than those stored for the other times although the highest proportion of large calluses (+++, ++++) were found on plants which had been inoculated immediately. Over 40% of the plants stored at 4°C for seven days showed small amounts of proliferation but no large calluses were formed. However the plants inoculated after this length of storage were generally much older than those held for shorter periods and were therefore less likely to react.

Most of the hypocotyls inoculated with sterile nutrient broth died, although in a few cases very small calluses were formed.

Although a large amount of work has been published on the effects of frost on plants (Levitt 1956), the writer has not found any reports on the response of plants to low temperatures just above freezing point. It was initially assumed that such temperatures

would slow up the physiological processes normally active in plants and in this way retard growth. But this experiment indicated that over short periods of up to one week, plants appear to age as if they were growing normally. The reactions of cold-treated plants to infection with E. coli were apparently the same as those of more slowly growing plants of the same age.

The conclusion drawn from this experiment was that holding seedlings at 4° C was not detrimental to the callus-forming capacity of the hypocotyls unless plants were kept for so long that their aging processes had carried them past the stage of maximum response. This maximum occurred in seedlings inoculated three to five days after planting. In practice it was found that hypocotyls stored for three days or longer became slightly flaccid, and were more difficult to decapitate without damaging the hypocotyl than were plants which had not been so treated.

Only 42% of all the hypocotyls inoculated with E. coli in this experiment showed any callusing, and of these only 9% proliferated to give large calluses. These percentages were considered too low for experimental work with E. coli on decapitated sunflower hypocotyls.

EXPERIMENT 4. A COMPARISON BETWEEN THE GROWTH OF INOCULATED DECAPITATED SEEDLINGS AND 1 MM HYPOCOTYL DISKS

De Ropp (1947a) grew 3 mm-long sections of the first internode of the sunflower which had been stripped of epidermis on media containing different concentrations of growth substances. Later

he grew 5 mm segments of sunflower hypocotyl on a sucrose-mineral agar and inoculated them with A. tumefaciens (de Ropp 1951c). He then noted the effect of inoculation on the fresh and dry weights of the segments, on the form of proliferation and on the extent of root formation.

This latter method seemed suited to the present investigations. With decapitated seedlings, the materials acting at the cut surface were presumably different from those taken up by the roots from the sucrose-mineral agar, because many changes would occur over the length of the seedlings. The reactions of the tissues at the cut surface would also be moderated by influences from adjacent tissue. By using de Ropp's method and reducing the size of the segments to 1 mm thick disks there would be a less complex system so that the effects of the bacteria and the composition of the medium on the tissues could be more easily studied.

An experiment was designed to compare the reactions of decapitated hypocotyls with 1 mm-thick hypocotyl disks, when both were infected with E. coli, A. tumefaciens and B. megaterium. Calluses induced by E. coli were reported to be similar to crown gall tumours (Philipson and Sheat 1963) but in the present experimental work, no observations had as yet been made on the growth of decapitated hypocotyls inoculated with A. tumefaciens. A comparison between these two bacteria-induced calluses was necessary to verify the reported similarity. Bacillus megaterium was the only other known non-pathogenic bacterium reported to induce cell division in plant tissues. It was of interest to determine the way in which

this reacted with sunflower tissue, it having previously been tested only with Vitis spp. (Fallot 1958).

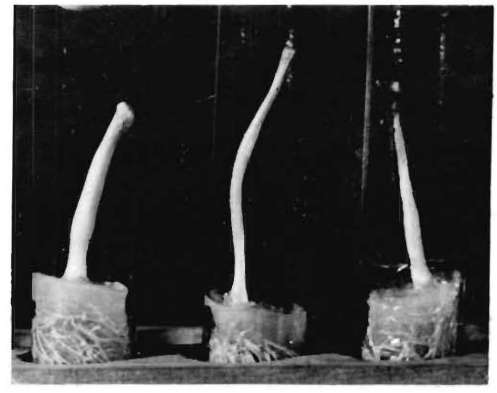
3.7 Experimental Details.

Three hundred sterilized seeds were planted in tubes containing 25 ml of de Ropp's medium, and were germinated and grown in the growth cabinet with low light intensity in order to obtain straight growth of the developing hypocotyls. Of these seedlings, 113 were decapitated and inoculated with 24-hour cultures of E. coli, A. tumefaciens, B. megaterium, or with sterile nutrient broth. During the preparation of E. coli cultures it was noted that the turbidity after 24 hours was similar to that after 48 hours and since younger cultures were desirable, inoculations were with 24-hour cultures in this and subsequent experiments. The inoculated seedlings were grown in high light intensity, low light intensity or in darkness.

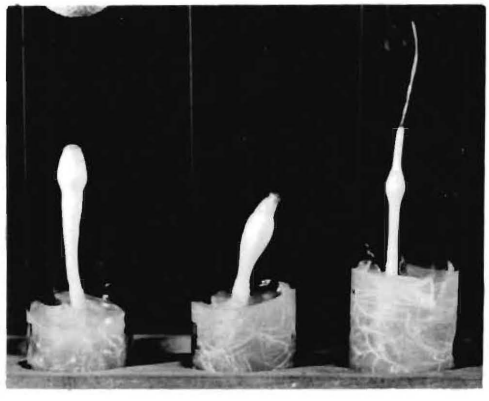
From the remaining 187 seedlings, 114 healthy plants were selected. Each hypocotyl was cut into 20 1 mm-thick disks which were placed on 50 ml of de Ropp's agar in a 100 ml Erlenmeyer flask. Each hypocotyl divided into 20 disks constituted one replicate. The disks were inoculated in the same way as decapitated hypocotyls, and were similarly distributed among the three light intensities. The majority of the seedlings had been stored at $4^{\circ}C$ for up to five days. This was necessary as the plants had matured at about the same time and could not all be treated owing to the numbers involved. From the total of 114 flasks of hypocotyl disks prepared, only 36 were harvested for fresh and

TABLE 3.5 RESPONSE OF HYPOCOTYLS TO INOCULATION

TREATMENT	NUMBER OF HYPOCOTYLS						
	POSITIVE RESPONSE				NO REACTION	DEAD	TOTAL
	+	++	+++	++++			
DARK							
Nutrient broth	1				2	7	10
E. coli	8	1			3	26	38
A. tumefaciens						5	5
B. megaterium						2	2
LOW LIGHT							
Nutrient broth						3	3
E. coli	2		1		3	4	10
A. tumefaciens	1					1	2
B. megaterium	1						1
HIGH LIGHT							
Nutrient broth	1		1			5	7
E. coli	8	3	2	4	3	10	30
A. tumefaciens		1		1		1	3
B. megaterium	1					1	2
TOTAL	23	5	4	5	11	65	113



a) Hypocotyls inoculated with nutrient broth and grown in high light. (Note callus formation in hypocotyl on the left.)



b) E.coli-inoculated hypocotyls grown in the dark.

c) E.coli-inoculated hypocotyls grown in high light.

d) A.tumefaciens-inoculated hypocotyls grown in high light.

FIGURE 3.2 SWELLING AND CALLUSING IN INOCULATED HYPOCOTYLS (Mag. x 0.54)

dry weight analysis because the remainder were contaminated. Although a large proportion of the flasks in this experiment were contaminated, this was substantially reduced in subsequent experiments by refinements in the experimental technique.

Results and Discussion.

3.8 Response of Decapitated Hypocotyls to Inoculation.

The decapitated hypocotyls reacted similarly to those described in previous experiments, and were recorded in the same way. (Table 3.5).

Under the three conditions of growth, the majority of hypocotyls inoculated with sterile nutrient broth died. Only in high light was one uninfected hypocotyl stimulated to produce a callus which was larger than the normal wound response (Figure 3.2a). Some of the other surviving hypocotyls showed a little swelling. Hypocotyls inoculated with A. tumefaciens showed rather surprising results in that only one hypocotyl, out of a total of ten grown under different conditions, proliferated with the formation of the usual crown gall callus. This particular seedling was grown in high light (Figure 3.2d). The large amount of proliferation of hypocotyls infected with E. coli and grown in high light (Figure 3.2c) was directly contrary to the results obtained in Experiment 1, when no proliferation above the normal wound response was observed in high light. Likewise the dark-grown plants inoculated with E. coli showed little proliferation compared with a large amount in the previous experiment. In the present case however, large swellings did form on the

infected plants (Figure 3.2b).

Although Fallot (1958) found that B. megaterium could induce proliferation of vine tissue, only slight callusing occurred in sunflower hypocotyls inoculated with this bacterium.

3.9 Response of Hypocotyl Disks to Inoculation.

Sunflower hypocotyl disks subjected to the same treatments and growing conditions as the decapitated hypocotyls, reacted differently. Although only a small number of disks was harvested, each treatment showed definite features of proliferation and root formation. Marked differences were evident between the treatments.

a) Morphology of Disks Grown in High Light Intensity. Uninfected disks grown in high light were green and appeared to be actively dividing over the three week period. In each disk a little callus was formed from the pith and vascular tissue on the upper surface. A few disks formed one or two short roots after two week's growth (Figure 5.7a). The disks inoculated with E. coli showed a large amount of proliferation from the lower surface after only one week's growth, while a week later cell division and expansion was so great that the epidermis was pushed at right angles to its original position. (Figure 5.7c). In the first week after inoculation, up to three roots were formed from many of the disks; these roots increased to 4 or 5 cm in length a week later. Disks infected with A. tumefaciens were pale green in colour and had proliferated from the upper surface after two weeks of growth (Figure 5.7e).

De Ropp's medium did not support the growth of E.coli, but in contrast, allowed prolific growth of A. tumefaciens. The viscous

fluid comprising the bacteria tended to swamp inoculated plant tissue. Gautheret (1959) also noted this growth and for this reason, the bacteria were eliminated from his cultures of crown gall tumour cells under-going subculturing, because they eventually killed the tissue if left to divide freely.

b) Morphology of Disks Grown in Low Light Intensity. In the low light uninfected disks proliferated a little from the upper surface. Many long roots with laterals were produced after two weeks of growth. In this respect they differed from those grown in the high light, when only occasional disks formed one or two short roots. Disks inoculated with E. coli produced a variable response in this light intensity. There was generally no surface proliferation and very little expansion of the tissue. The disks generally became brown before finally dying and in most, no roots were formed. A. tumefaciens-infected disks were similar morphologically to those grown under the high light conditions.

c) Morphology of Disks Grown in the Dark. Hypocotyl disks inoculated with sterile nutrient broth and grown in the dark showed a small amount of proliferation from the upper surface, and a large number of long roots was formed after two weeks. Disks inoculated with E. coli were mainly brown and necrotic after one week, and no roots were formed. Disks inoculated with A. tumefaciens were similar to those grown in the light although the proliferating tissue was white.

3.10 A Comparison of the Growth of Hypocotyl Disks in the Three Light Conditions.

Uninfected disks in all light intensities proliferated only a little from the surface tissue. Many roots formed from disks grown in low light and the dark but few were formed in the high light. There was an interaction between the effects of inoculation with E. coli and light of differing intensities, so that growth of the inoculated disks was stimulated in high light, and inhibited in low light and the dark, compared with the uninfected controls. There was little difference in the form of A. tumefaciens-inoculated disks grown in the three different light conditions.

3.11 Morphology of Disks Inoculated with B. megaterium and Grown in the Three Light Conditions.

Disks inoculated with B. megaterium were not harvested, but from observation it was apparent that in all light conditions little growth occurred before the tissues became brown. Disks grown in the dark enlarged more when inoculated with B. megaterium than when inoculated with E. coli. No roots were formed under any of the light conditions. Artichoke tissue cultured by Fallot (1964) in the presence of B. megaterium was stimulated to form abundant callus. He found that few roots were formed from the tissue, and that the large increase in fresh weight of the cultures was not paralleled by a dry weight increase. This contrasts with the present findings for sunflower tissue.

3.12 Analysis of the Weights of Hypocotyl Disks.

Each treatment contained a variable number of replicates, each

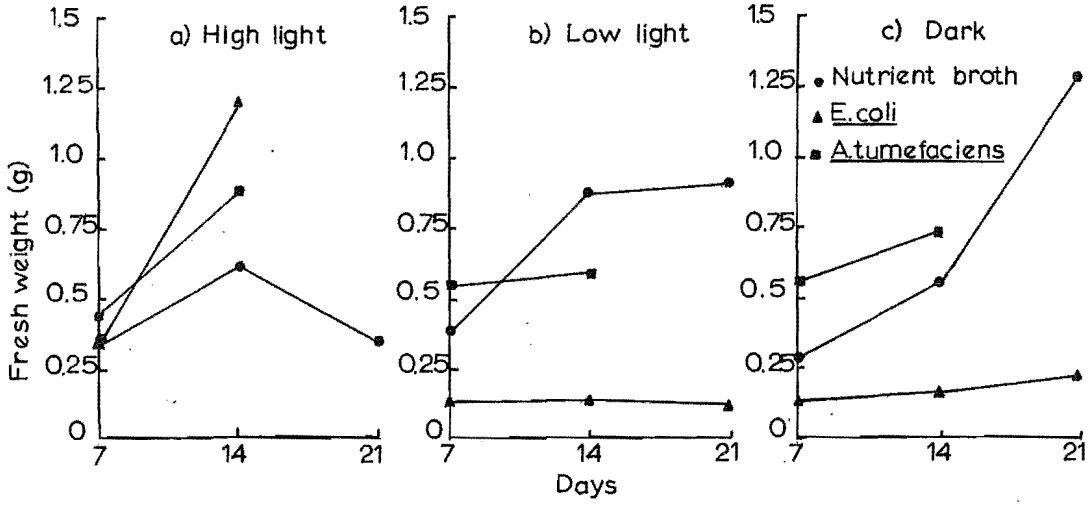


FIGURE 3.3a,b,c. EFFECT OF BACTERIAL TREATMENT ON DISKS GROWN IN DIFFERENT LIGHT INTENSITIES

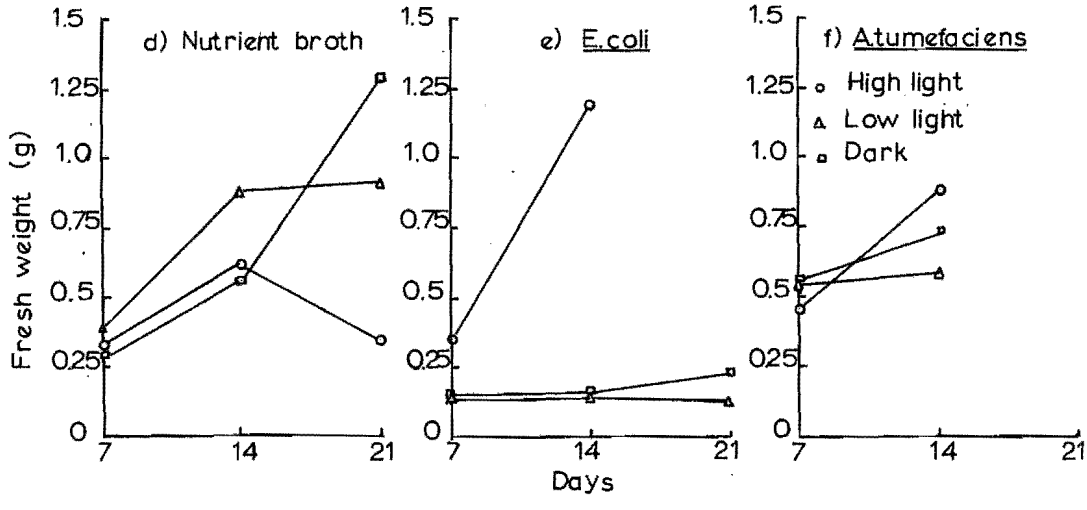


FIGURE 3.3d,e,f. EFFECT OF LIGHT ON GROWTH OF INOCULATED DISKS

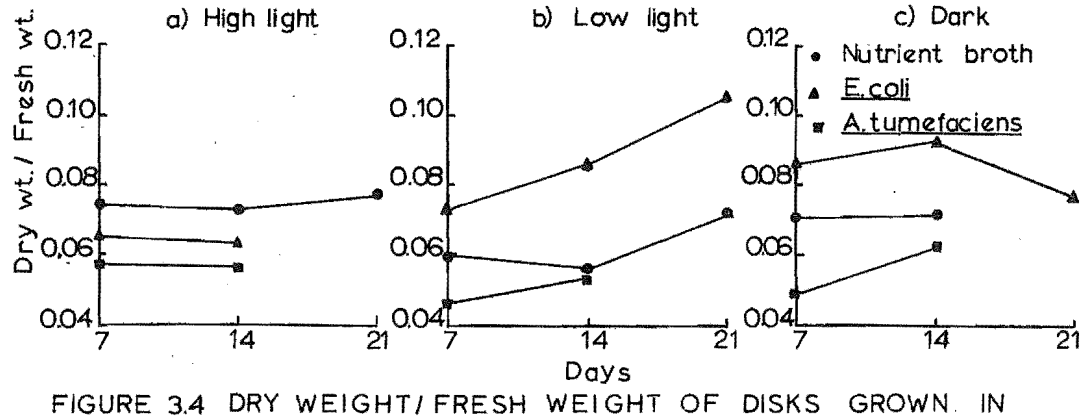


FIGURE 3.4 DRY WEIGHT/ FRESH WEIGHT OF DISKS GROWN IN DIFFERENT LIGHT INTENSITIES

one of which consisted of one hypocotyl divided into 20 disks. (Fresh and dry weights are recorded in Table 1 Appendix I). The average fresh weights of the replicates for each treatment were graphed, showing the variations between the bacterial treatments in each light intensity (Figure 3.3a, b, c), as well as the variations within each bacterial treatment with growth in different light intensities (Figure 3.3d, e, f.).

The trends observed in the fresh weights of the disks could be expected from their morphological forms. After one week's growth, disks inoculated with A. tumefaciens were the heaviest in all light conditions (Figure 3.3a, b, c). At this time, few of the disks, either infected or uninfected, had formed roots, so that the fresh weights were a measure of proliferation of the disks, rather than of their ability to form roots. After two weeks of growth, disks inoculated with E. coli and grown in high light were heavier than uninfected disks (Figure 3.3a), while in low light and the dark, the growth of E. coli-inoculated disks was markedly inhibited, both in comparison with uninfected disks, and with infected disks grown in high light (Figure 3.3b, c, e). After three weeks E. coli-infected disks were again much lighter than uninfected disks (Figure 3.3b,c). Over the period of growth the weights of these disks increased only a little in comparison with those grown in high light. Large weight increases occurred with uninfected disks in low light and the dark, in the third week of growth, because of formation of roots. In high light, uninfected disks formed only a few roots, with a correspondingly lower weight

(Figure 3.3d). Light had only a minor effect on the growth of disks infected with A. tumefaciens, those grown in high light being slightly heavier after two weeks (Figure 3.3f).

For each bacterial treatment, there was a close relationship between fresh and dry weights, when differences in light treatment were neglected. Correlation coefficients were found for these. They were 0.979 for uninfected disks, 0.993 for disks inoculated with E. coli, and 0.950 for A. tumefaciens-inoculated disks.

The ratio of dry weight to fresh weight for each treatment is shown in Figure 3.4a,b,c (and in Table 2 Appendix I). When tissues divide, increase in fresh weight is paralleled by an increase in dry weight, but when only cell expansion occurs by water uptake, the increase in fresh weight is not paralleled by a corresponding dry weight increase. Where the dry weight to fresh weight ratio remains constant, it can be assumed that growth of the tissues by both cell division and expansion has been normal, but where the ratio decreases, increased cell expansion and water uptake have occurred in the tissues. Where the curve for the ratio increases it can be assumed that growth by cell division has taken place which may have been followed by lignification.

In all light intensities, disks inoculated with A. tumefaciens showed the lowest dry weight to fresh weight ratio. This indicated that a higher uptake of water had occurred in these disks than in uninfected or E. coli-inoculated disks. For disks grown

in high light, those inoculated with E. coli had the highest fresh weights, although they showed rather less water uptake in proportion to this weight than A. tumefaciens-inoculated disks (Figure 3.4a). In low light and the dark, disks inoculated with E. coli had the least amount of water uptake. Under these conditions, the disks grew only a little, and it may be assumed that the increase in fresh weight was due more to cell division and resulting lignification, than to water uptake. In high light, then, cells in E. coli-infected disks were stimulated to take up water as well as to divide, but in low light and the dark, mainly cell division occurred.

3.13 Conclusion.

The results obtained from these treatments with only small replicate numbers of 1 mm hypocotyl disks, showed that a quantitative method had been found by which the effects of different bacterial treatments on sunflower tissues could be studied. By this method, the extent of tissue proliferation, as well as water uptake and cell expansion, or cell division, could be measured. The experiments with decapitated hypocotyls were unreliable, since the reactions had to be recorded subjectively. In addition, these reactions were tempered by the greater influence of neighbouring tissues. With hypocotyl disks only 1 mm thick, very little food reserve was present in each segment at the time of inoculation, so that the results were more directly a reflection of the interaction of bacteria with tissue supplied with chemically defined nutrients.

With hypocotyl disks the tissue response to infection with

E. coli could be distinguished from the normal wound reaction of uninfected tissues. Hence results were more definite than those obtained by using decapitated sunflower seedlings. E. coli-inoculated disks reacted quite differently from decapitated hypocotyls inoculated in the same way. Further, the action of E. coli on hypocotyl disks presented an interesting problem in the interaction of light with the bacterial treatment.

EXPERIMENT 5. EFFECTS OF DIFFERING LIGHT TREATMENTS ON INOCULATED DECAPITATED SEEDLINGS AND HYPOCOTYL DISKS

The previous experiment indicated that E. coli-infected disks reacted differently when grown in different light conditions. The present experiment was designed to investigate this further. Disks grown in three light conditions (high light, low light, and dark) were prepared from seedlings grown in each of the three conditions, giving nine possible combinations of seedling-light and disk-light treatments. This would determine whether the growth conditions of the seedlings in any way influenced the subsequent growth of the hypocotyl disks. A further comparison was made between the growth of decapitated hypocotyls, and hypocotyl disks, treated in the same way.

3.14 Experimental Details.

Seeds were sterilized and planted in de Ropp's agar medium and grown under conditions of either high or low light intensity or in the dark. When these seedlings were 3 to 4 cm high, they were decapitated and inoculated with either sterile nutrient broth, or nutrient broth cultures of E. coli or A. tumefaciens. The

decapitated seedlings were replaced in the growth cabinets in such a way that every combination of light treatment of seedlings and hypocotyls occurred. Twenty replicates of each treatment in which hypocotyls were inoculated with E. coli were prepared, while there were ten replicates of uninfected hypocotyls and two to four replicates of A. tumefaciens-inoculated hypocotyls. The reactions of the hypocotyls to the treatments were described three to four weeks after inoculation.

Seeds, which after germination and growth were to be cut into disks, were sterilized and planted at intervals so that when they matured they could be treated immediately. The seedlings were grown under the three different lighting conditions, as in the first part of the experiment, and after cutting the hypocotyls into disks and inoculating them, the disks were again distributed in the different light intensities so that all nine combinations of light treatment of seedlings and disks occurred. Each hypocotyl constituted one replicate, and was divided into ten disks. Generally, sixteen replicates of each light treatment of disks inoculated with E. coli were prepared. Only eight replicates of each light treatment were inoculated with A. tumefaciens or sterile broth. Disks one, two, three and four weeks old were harvested so that four replicates of E. coli-inoculated disks were harvested each week, and two replicates of A. tumefaciens and sterile broth-inoculated disks. Both the fresh and the dry weights of the disks were recorded in Table 3 Appendix I.

TABLE 3.6 RESPONSE OF HYPOCOTYLS TO INOCULATION

TREATMENT		NUMBER OF HYPOCOTYLS									
BACTERIAL	LIGHT	POSITIVE RESPONSE								NO REACTION	DEAD
		SWELLING				PROLIFERATION					
		+	++	+++	++++	+	++	+++	++++		
E. coli	High light	20	7	21		2				3	7
	Low light	10	25	5		12				6	1
	Dark	16	11			18				15	
Nutrient broth	High light	5		11	6	5				1	2
	Low light	3	12		5	8				2	
	Dark	10				9				11	
A. tumefaciens	High light							1	8		
	Low light						1	3	4		
	Dark	4				1		1	3		

TABLE 3.7 RESPONSE OF HYPOCOTYLS INOCULATED WITH A.TUMEFACIENS

LIGHT TREATMENTS OF SEEDLINGS		NUMBER OF HYPOCOTYLS			
BEFORE INOCULATION	AFTER INOCULATION	SWELLING	PROLIFERATION		
		+	+	++	+++ ++++
High light	High light				3
	Low light				2
	Dark				3
Low light	High light				2
	Low light			1	2
	Dark				1 2
Dark	High light				2
	Low light	2			1
	Dark	2	1		1

Results and Discussion

3.15 Response of Decapitated Hypocotyls to Inoculation.

The reactions of decapitated hypocotyls inoculated with the various bacteria are recorded in Table 3.6. Because of the pronounced swelling that occurred in many hypocotyls this response is tabulated separately from that of callusing. The degree of swelling is also shown. Seedling light treatment had little effect on the subsequent growth of uninfected or *E. coli*-inoculated hypocotyls and is therefore not differentiated in the table.

Of all the treated hypocotyls, only those infected with *A. tumefaciens* showed any marked proliferation. In the case of hypocotyls inoculated with *E. coli* or sterile broth, callusing, where present, was very slight, although all the hypocotyls showed a tendency to swell just below the cut surface. Fewer hypocotyls died than in earlier experiments, presumably because better techniques of seed sterilization had been developed (see Chapter 2). The few deaths recorded were mainly in the hypocotyls grown in the high light, where conditions in the tubes were drier than in the low light or the dark. In all treatments more callusing or swelling occurred when the hypocotyls were grown in high or low light.

The influence of seedling light treatment on the subsequent development of inoculated hypocotyls was evident only on inoculation with *A. tumefaciens*. When the treated hypocotyls were grown in the dark most of those from seedlings grown in the low light or dark swelled only a little, while those from seedlings grown in high light showed the expected response of crown gall formation

(Tables 3.6, 3.7). All hypocotyls inoculated with A. tumefaciens and grown in the low light or the high light formed large galls, independent of the seedling light treatment before inoculation. However, only small replicate numbers were used for inoculation with A. tumefaciens, so that no definite conclusions on the effects of light could be drawn from the results. De Ropp (1948a) found that light had no influence on the number of inoculated plants forming crown galls. Similarly de Capite (1955) showed that below 26°C light had little effect on the growth of bacteria-free crown gall tissue, but above this temperature growth was greatly increased. In the present experimental work where plants were grown at 25°C ± 2°, fewer dark-grown than light-grown hypocotyls formed crown galls. It is apparent that different tissues react to the bacteria in different ways, depending on the conditions under which they are grown.

3.16 Response of Hypocotyl Disks to Inoculation.

More satisfactory results were obtained from the inoculation of hypocotyl disks grown under the same conditions as the decapitated hypocotyls just described. The gross morphological responses of the disks to the bacterial and light treatments were similar to those described in the previous experiment, but with increased replication, more definite trends could be defined. These are described below. (The responses of the disks have been recorded by means of symbols in Table 4, Appendix I).

a) Morphology of Uninfected Disks Grown under Different Conditions.

Most uninfected disks proliferated a little from the upper or lower

surfaces, or both, after one week's growth, regardless of the light conditions in which they were grown. At this age, disks grown in the high or low light produced few if any roots when the seedlings from which they were prepared had developed in the light. When the seedlings were grown in the dark more roots formed from the light-grown disks. When the disks were grown in the dark, roots were formed regardless of the light treatment of the seedlings from which they were prepared. It appeared that growth of the seedlings in the light had an inhibitory effect on root formation in the high light-grown disks prepared from them.

Growth of the uninfected disks two, three and four weeks old continued in much the same way as disks one week old. However, the inhibitory effect of seedling-light treatment on root growth of light-grown disks was not as marked, since more disks formed roots.

Scattered among the more normal types of uninfected disks grown in the low light or the dark, were a few which had expanded to a much greater extent than usual. These disks quickly became brown and appeared to be hyperhydric. Disks reacting in this way usually did not form roots.

b) Morphology of Disks Inoculated with E. coli and Grown under Different Conditions. As described in Experiment 4, disks inoculated with E. coli showed varying morphological responses depending on the light conditions under which they were grown. The different responses were apparent after only one week's growth. The majority of disks grown in high light proliferated from the lower surface,

pushing the epidermis out at right angles to its usual position. Only occasional very short roots were formed during the first week after inoculation. In contrast to this, most disks grown in the low light and the dark showed no signs of proliferation, although some growth had occurred, as was evident by the increased size of the disks. In a number of cases the disks died, particularly when they had been grown in the dark. However a few disks infected with E. coli and grown in the dark proliferated, and were similar to infected disks grown in the high light.

The difference in response between disks grown in the high light, and those grown in the low light and the dark, persisted through the four weeks of growth of the disks. During this time, high light-grown disks increased in size and continued to proliferate from the lower surface and form more roots. Low light and dark-grown disks formed occasional short roots, but these ceased growing when the disks became necrotic. In contrast to uninfected disks grown in the high light, those infected with E. coli and grown under the same conditions, were not apparently influenced by the light treatment of the seedlings from which the disks were prepared.

c) Morphology of Disks Inoculated with A. tumefaciens and Grown under Different Conditions. In all three light conditions, disks infected with A. tumefaciens proliferated mainly from the upper surface where the bacteria had been placed. The tissues formed were compact and firm. Greater growth was obtained in high light-grown disks than in those grown in the low light or the dark. The

bacteria themselves grew well on de Ropp's medium, and in the dark, frequently divided so much that they covered the disks in a viscous fluid which limited growth. After one week's growth only a few of the disks had formed short roots with blackened tips. A few more roots were formed during the remaining weeks before harvesting, but in all cases their growth was slow. Again, as with disks inoculated with E. coli, the light treatment of the seedlings did not apparently influence the morphology of A. tumefaciens-infected disks.

3.17 Analysis of the Weights of Hypocotyl Disks.

Because unequal numbers of replicates were used for each treatment, the average treatment weights were used for comparison. The average fresh and dry weights of each treatment are recorded in Tables 3.8-3.14. Each fresh weight in the tables represents the average of a number of replicates, each containing ten disks. The fresh weights of the hypocotyls were statistically analysed using the analysis of variance test. Variance is a measure of the deviation of samples from the mean of a distribution (Weatherburn 1957). The analysis of variance test has been described by Fisher (1938) as the "separation of the variance ascribable to one group of causes from the variance ascribable to other groups." The methods of calculation used in analysing the data are described by Steel and Torrie (1960). For ease in calculation the fresh weight x 10^4 was used as the basic unit.

Because of interaction among the treatments, no simple differences occurred within the main treatments. It could not

TABLE 3.8a. AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND E. COLI ONE WEEK AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient broth	HL*	1584	1202	1303	4089
	LL	1792	1212	2341	5345
	D	1542	1477	2293	5312
	HL + LL + D	4918	3891	5937	14746
E. coli	HL	2409	1176	889	4474
	LL	1641	1192	818	3651
	D	1578	1206	1148	3932
	HL + LL + D	5628	3574	2855	12057
Broth + E. coli	HL	3993	2378	2192	8563
	LL	3433	2404	3159	8996
	D	3120	2683	3441	9244
	HL + LL + D	10546	7465	8792	26803

* HL = High light LL = Low light D = Dark

TABLE 3.8b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.*	SUM OF SQUARES	MEAN SQUARE	F
Bacteria	1	401706	401706	8.55* at 5%
Seed light	2	39597	19799	NS.
Disk light	2	796111	398056	8.47* at 5%
B x SL	2	418670	209335	4.45* at 10%
B x DL	2	1282179	641090	13.64 at 2.5%
SL x DL	4	613712	153428	3.26 NS.
Error	4	188039	47010	
Total	17	3740014		

* D.F. = Degrees of Freedom

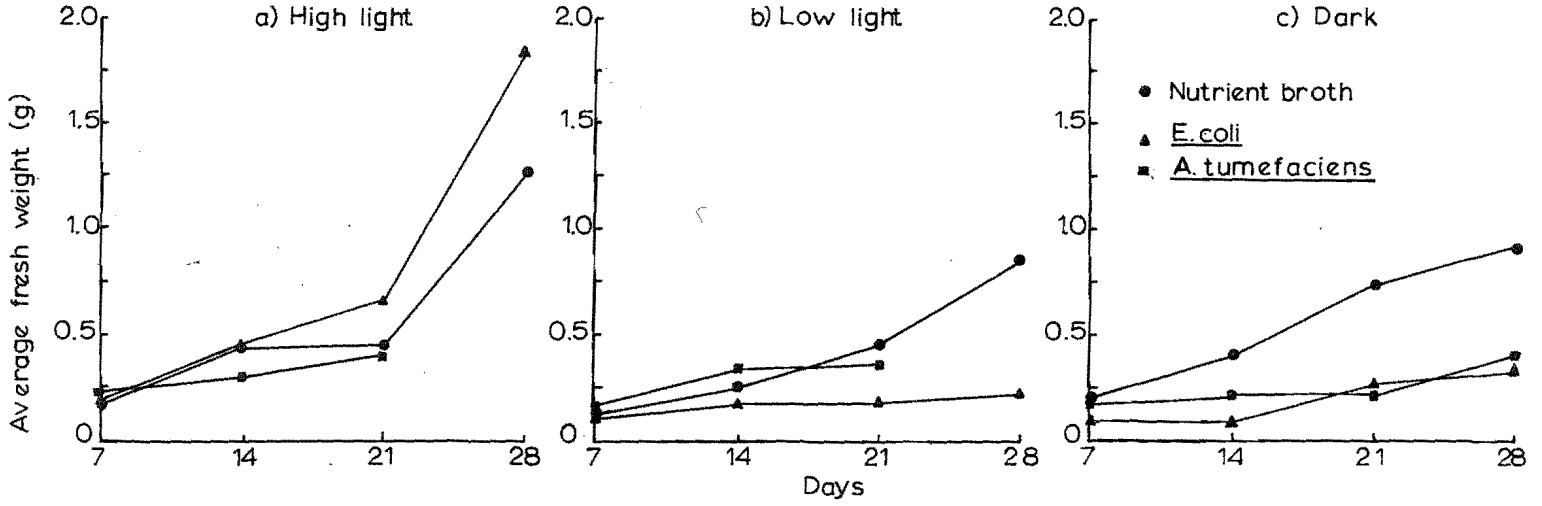


FIGURE 3.5 AVERAGE FRESH WEIGHTS OF DISKS GROUPED ACCORDING TO DISK-LIGHT AND BACTERIAL TREATMENTS

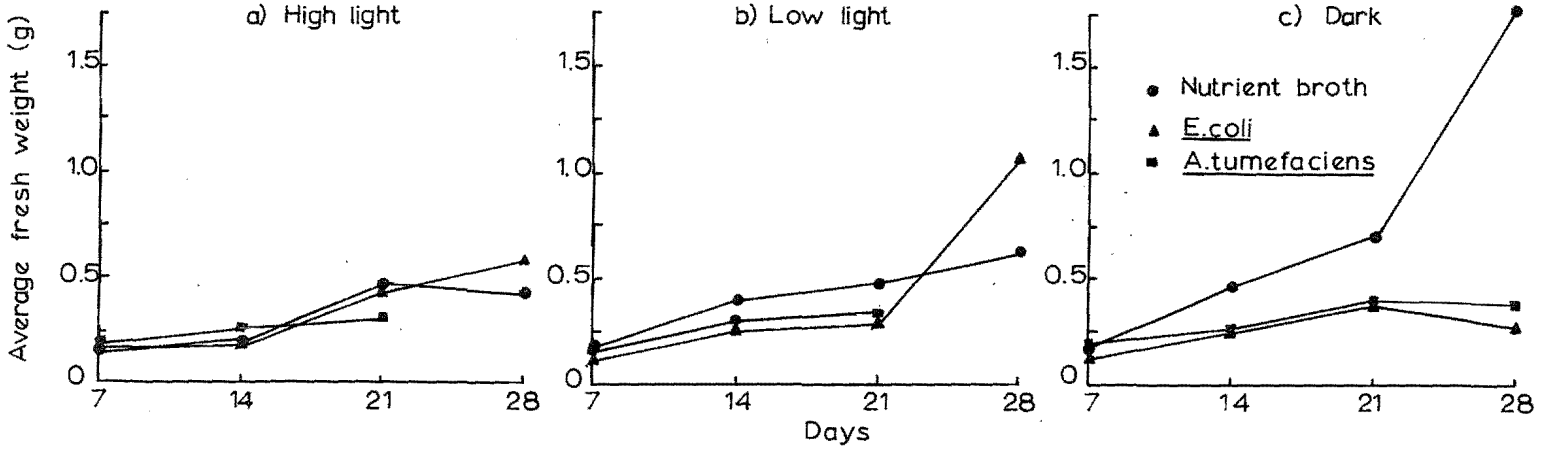


FIGURE 3.6 AVERAGE FRESH WEIGHTS OF DISKS GROUPED ACCORDING TO SEEDLING-LIGHT AND BACTERIAL TREATMENTS

be said, for example, that growth of all disks was stimulated in high light, or that one bacterial treatment stimulated growth above all the others, under all conditions.

a) Analysis of Variance for Disks One Week Old. For uninfected and E. coli-inoculated disks, an interaction occurred between the bacterial and disk-light treatments (Table 3.8a, b). When grown in the dark, uninfected disks were much heavier than those inoculated with E. coli (Figure 3.5c). In addition, dark-grown uninfected disks were significantly heavier than those grown in the light (Figure 3.5a,b,c). E. coli-infected disks were differentially stimulated by the light treatment to an even greater extent, and in a different direction, than uninfected disks, so that those grown in the dark and low light were much lighter than disks grown in high light (Figure 3.5a,b,c). The fresh weights of E. coli-inoculated disks were heavier than uninfected disks in high light, and much lighter in the dark.

An interaction (significant at the 10% level) was also found among the bacterial and seedling-light treatments. Uninfected disks prepared from seedlings grown in low light and darkness were heavier than those inoculated with E. coli (Figure 3.6a,b,c). Morphological observations showed that the uninfected disks formed more roots under these conditions.

No significant differences were found between the fresh weights of uninfected and A. tumefaciens-inoculated disks although the latter were heavier when grown in high light than in low light or darkness (Table 3.9). Much of the weight increase in unin-

TABLE 3.9 AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND A. TUMEFACIENS ONE WEEK AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient broth	HL	1584	1202	1303	4089
	LL	1792	1212	2341	5345
	D	1542	1477	2293	5312
	HL + LL + D	4918	3891	5937	14746
A. tumefaciens	HL	1886	1955	1314	5155
	LL	2311	1437	1543	5291
	D	2397	1095	2039	5531
	HL + LL + D	6594	4487	4896	15977
Broth + A. tumefaciens	HL	3470	3157	2617	9244
	LL	4103	2649	3884	10636
	D	3939	2572	4332	10843
	HL + LL + D	11512	8378	10833	30723

TABLE 3.10a AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND E. COLI TWO WEEKS AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient Broth	HL	1625	2339	2514	6478
	LL	5087	2214	4512	11813
	D	5944	2847	5126	13917
	HL + LL + D	12656	7400	12152	32208
E. coli	HL	2912	1666	861	5439
	LL	5049	1833	905	7787
	D	4767	1709	842	7318
	HL + LL + D	12728	5208	2608	20544
Broth + E. coli	HL	4537	4005	3375	11917
	LL	10136	4047	5417	19600
	D	10711	4556	5968	21235
	HL + LL + D	25384	12608	14760	52752

TABLE 3.10b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Bacteria	1	7558272	7558272	31.76 ***
Seed light	2	8251491	4125746	17.33*at 2.5%
Disk light	2	15595925	7797963	32.76 ***
B x SL	2	2580894	1290447	5.42*at 10%
B x DL	2	8424726	4212363	17.70*at 2.5%
SL x DL	4	5341703	1335426	5.61*at 10%
Error	4	952003	238001	
Total	17	48705014		

TABLE 3.11a AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND A. TUMEFACIENS TWO WEEKS AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient Broth	HL	1625	2339	2514	6478
	LL	5087	2214	4512	11813
	D	5944	2847	5126	13917
	HL + LL + D	12656	7400	12152	32208
A. tumefaciens	HL	2421	3324	2344	8089
	LL	3706	3439	1870	9015
	D	3135	2755	2346	8236
	HL + LL + D	9262	9518	6560	25340
A. tumefaciens + Broth	HL	4046	5663	4858	14567
	LL	8793	5653	6382	20828
	D	9097	5602	7472	22153
	HL + LL + D	21918	16918	18712	57548

TABLE 3.11b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Bacteria	1	2620524	2620524	6.99*at 10%
Seed light	2	5472397	2736199	7.30*at 5%
Disk light	2	2138715	1069358	2.85 N.S.
B x SL	2	4495791	2247896	6.00*at 10%
B x DL	2	5208747	2604374	6.95*at 5%
SL x DL	4	4243768	1060942	2.83 N.S.
Error	4	1498956	374739	
Total	17	25678898		

fected disks was due to the growth of roots, while in disks inoculated with A. tumefaciens, proliferation of the hypocotyl tissues occurred and fewer roots were formed, so that the gross morphology of these disks was quite different although there were no significant differences in fresh weights.

b) Analysis of Variance for Disks Two Weeks Old. Greater differences were apparent among treated disks two weeks old. (Table 3.10a, b). In both low light and darkness the growth of disks inoculated with E. coli was very much inhibited compared with that in high light (Figure 3.5a,b,c), so that a more highly significant interaction between bacterial and disk-light treatments was found compared with that for disks one week old. Uninfected disks grown in low light were lighter than those grown in high light or darkness, thus differing from disks one week old. There was no morphological explanation for this. However, E. coli-inoculated disks were slightly heavier in high light, and very much lighter in low light or the dark than uninfected disks, thus maintaining the same relationship found after one week's growth (Figure 3.5a,b,c).

Although disks inoculated with A. tumefaciens were heavier when grown in high or low light than in the dark, the weights were not significantly different (Figure 3.5a,b,c) (Table 3.11a, b). In both high light and the dark the infected disks were significantly lighter than uninfected disks.

An interaction found between seedling-light and bacterial treatments in the comparisons of both E. coli and A. tumefaciens-

TABLE 3.12a AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND E. COLI THREE WEEKS AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient broth	HL	4015	4390	5162	13567
	LL	2730	4271	7298	14299
	D	6489	4794	9643	20926
	HL + LL + D	13234	13455	22103	48792
E. coli	HL	4911	1625	6684	13220
	LL	5802	2291	849	8942
	D	9067	1702	754	11523
	HL + LL + D	19780	5618	8287	33685
Broth + E. coli	HL	8926	6015	11846	26787
	LL	8532	6562	8147	23241
	D	15556	6496	10397	32449
	HL + LL + D	33014	19073	30390	82477

TABLE 3.12b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Bacteria	1	12678969	12678969	2.08 NS
Seed light	2	7189979	3594990	NS
Disk light	2	18295075	9147538	1.50 NS
B x SL	2	6860076	3430038	NS
B x DL	2	36512788	18256394	2.99 NS
SL x DL	4	11948078	2987019	NS
Error	4	24414456	6103614	
Total	17	117899421		

TABLE 3.13a AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND A. TUMEFACIENS THREE WEEKS AFTER TREATMENT ($g \times 10^4$)

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient Broth	HL	4015	4390	5162	13567
	LL	2730	4271	7298	14299
	D	6489	4794	9643	20926
	HL + LL + D	13234	13455	22103	48792
A. tumefaciens	HL	3802	3498	2385	9685
	LL	3868	3919	2108	9895
	D	4497	3349	2500	10346
	HL + LL + D	12167	10766	6993	29926
Broth + A. tumefaciens	HL	7817	7888	7547	23252
	LL	6598	8190	9406	24194
	D	10986	8143	12143	31272
	HL + LL + D	25401	24221	29096	78718

TABLE 3.13b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Bacteria	1	19773664	19773664	27.09**at 1%
Seed light	2	6405880	3202940	4.39*at 10%
Disk light	2	2156169	1078085	1.48 N.S.
B x SL	2	4626593	2313297	3.17 N.S.
B x DL	2	19673221	9837611	13.48*at 2.5%
SL x DL	4	4096082	1024021	1.40 N.S.
Error	4	2919696	729924	
Total	17	59651305		

inoculated disks with uninfected disks showed that uninfected disks were much heavier when the seedlings were grown in low light or the dark than in high light (Figure 3.6b,c). This occurred because more roots were formed in the heavier disks. There was no similar interaction with seedling light treatment in the infected disks.

c) Analysis of Variance for Disks Three Weeks Old. After three weeks of growth, the total range of variability of all the treatments was so large that no significant differences were recorded among E. coli-inoculated and uninfected disks (Table 3.12a, b). However, the fresh weights of the disks followed the same trends found after one and two weeks of growth. Disks inoculated with E. coli were heavier than uninfected controls in high light, but were much lighter in low light and darkness (Figure 3.5a,b,c).

A significant interaction was recorded between uninfected disks and those inoculated with A. tumefaciens, when grown in different light conditions (Table 3.13a, b). In particular, infected disks grown in the dark were very much lighter than uninfected disks (Figure 3.5c).

d) Analysis of Variance for Disks Four Weeks Old. As with disks harvested after three weeks, those four weeks old showed a great range of variability among the treatments. Again, disks inoculated with E. coli were heavier than uninfected disks when grown in high light, and were much lighter in low light and the dark, although no significant interaction was found among the

TABLE 3.14a AVERAGE FRESH WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH NUTRIENT BROTH AND E. COLI FOUR WEEKS AFTER TREATMENT. ($g \times 10^4$).

BACTERIAL TREATMENT	SEED LIGHT TREATMENT	DISK LIGHT TREATMENT			
		HL	LL	D	HL + LL + D
Nutrient Broth	HL	3657	3387	5614	12658
	LL	2815	5599	10287	18701
	D	31884	16511	11556	59951
	HL + LL + D	38356	25497	27457	91310
E. coli	HL	10742	1761	3780	16283
	LL	23692	3778	4496	31966
	D	20843	1281	1401	23525
	HL + LL + D	55277	6820	9677	71774
Broth + E. coli	HL	14399	5148	9394	28941
	LL	26507	9377	14783	50667
	D	52727	17792	12957	83476
	HL + LL + D	93633	32317	37134	163084

TABLE 3.14b ANALYSIS OF VARIANCE

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Bacteria	1	21203072	21203072	4.88*at 10%
Seed light	2	251250877	125625439	7.53*at 5%
Disk light	2	387499575	193749788	4.50*at 10%
B x SL	2	231455928	115727965	2.67 N.S.
B x DL	2	137343423	68671712	1.76 N.S.
SL x DL	4	181543392	45385848	
Error	4	102940580	25735145	
Total	17	1313236901		

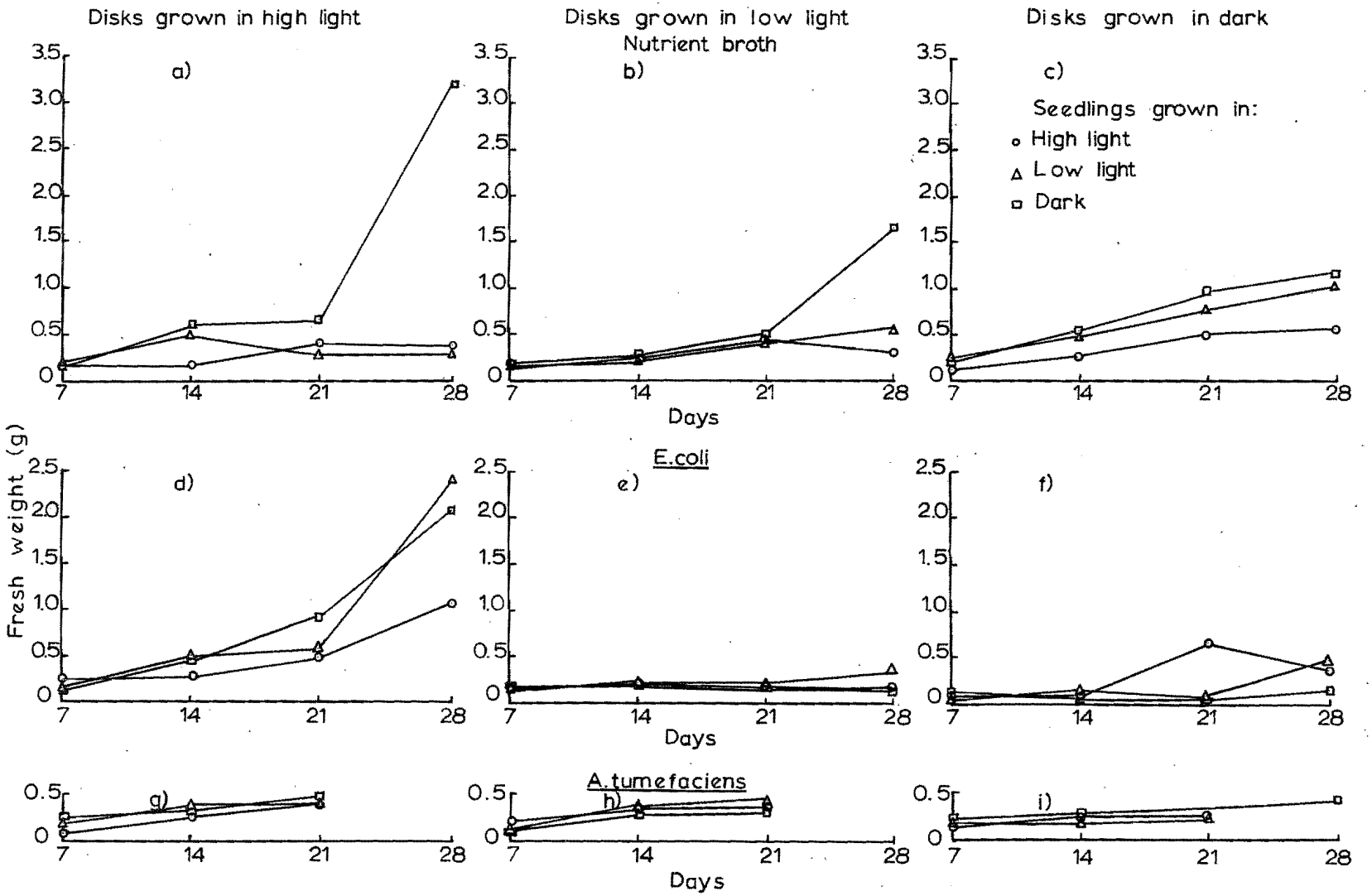


FIGURE 3.7 EFFECT OF DISK AND SEEDLING-LIGHT TREATMENTS ON FRESH WEIGHTS OF INOCULATED DISKS

bacterial and disk-light treatments (Figure 3.5a,b,c, Table 3.14a, b). The only significant interaction was between bacterial and seedling-light treatments. Uninfected disks prepared from seedlings grown in the dark were much heavier than E. coli-inoculated disks prepared from similarly treated seedlings (Figure 3.6c). They were also heavier than uninfected disks prepared from light-grown seedlings.

3.18 Discussion of the Analysis of Variance for Hypocotyl Disks of Different Ages.

The trends in root formation and tissue proliferation observed in the morphological responses of the hypocotyl disks, were verified by the statistical analyses carried out on the average fresh weights of the hypocotyl disks. Figure 3.7 shows the changes in weight of inoculated disks grown under different light conditions when the seedlings from which they were prepared had been grown in different conditions. Figures 3.5 and 3.6, previously referred to, show only certain aspects of these changes.

The main effect emerging from the analysis over the four weeks of growth was the interaction between E. coli-inoculated disks and the various light conditions in which they were grown. Growth was stimulated above that of uninfected disks in high light (Figure 3.7a, d), and was comparatively inhibited in both low light and the dark (Figure 3.7b, e, c, f). The growth of disks inoculated with A. tumefaciens was not as great as that of uninfected disks (Figure 3.7g,h,i). In the dark, growth of

infected disks was inhibited in comparison with those grown in low and high light. Thus light treatment of the disks elicited different responses from those inoculated with E. coli and A. tumefaciens. In contrast to infected disks, uninfected disks grown in the dark were heavier than those grown in low or high light (Figure 3.7a,b,c).

The effects of seedling light treatment on subsequent growth of the disks was most important in uninfected disks grown in high light. Those prepared from seedlings grown in the dark produced many more roots, with a resulting increase in weight, than disks from low and high light-grown seedlings (Figure 3.7a). Neither E. coli nor A. tumefaciens-infected disks were affected by the light treatment of the seedlings.

Thus, the main effect of the disk-light treatment was on differential stimulation of E. coli-infected disks, and the main effect of seedling-light treatment was on the differential stimulation of uninfected disks.

3.19 Analysis of Dry Weights of the Disks in Comparison with their Fresh Weights.

For all treatments, the correlation coefficients between dry and fresh weights of the disks were very high. The correlation coefficient for E. coli-inoculated disks grown in high light was 0.9938, and for uninfected disks in the same conditions, 0.9993. In order to determine whether disks treated in different ways responded by cell division or expansion through water uptake, the ratio of dry weight to fresh weight was found (as described in

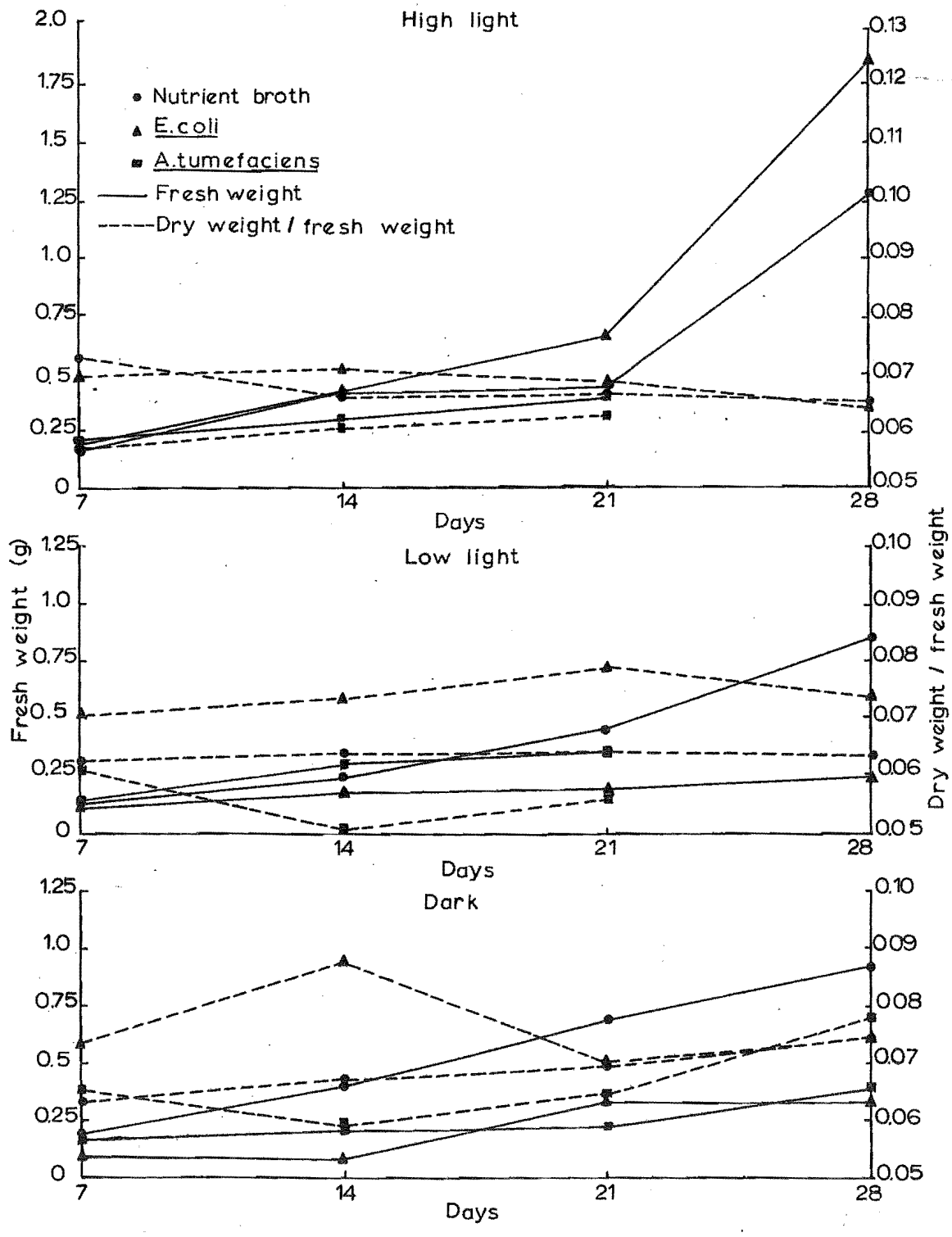


FIGURE 3.8 DRY WEIGHT/ FRESH WEIGHT GRAPHED AGAINST FRESH WEIGHT OF DISKS FOR DIFFERENT LIGHT TREATMENTS

Experiment 4). The seedling-light treatments were combined for each bacterial and disk-light treatment. The ratio was graphed against the average fresh weights of the disks so that variations between them could be noted (Figure 3.8).

a) Disks Grown in High Light. The curves for the dry weight to fresh weight ratio were similar for uninfected and E. coli-inoculated disks, showing that in both cases the amount of water uptake and of cell division were in the same proportion. Fresh weights of the infected disks were nevertheless higher than those of uninfected disks. In both uninfected and E. coli-inoculated tissues four weeks old, a sudden increase in fresh weight occurred, but the proportions of cell division and water uptake remained the same. Disks infected with A. tumefaciens showed more water uptake than either of the other two treatments.

b) Disks Grown in Low Light. The ratio for uninfected disks grown in the low light was similar to that for disks grown in high light. The dry weight to fresh weight ratio for disks inoculated with E. coli was much higher than that for uninfected disks grown under the same conditions. Morphological observations showed that these disks grew only a little, and it can be assumed, from the dry weight to fresh weight ratio, that the majority of this increase was due to cell division rather than expansion and water uptake. Disks inoculated with A. tumefaciens were more hyperhydric than those grown in high light.

c) Disks Grown in the Dark. Over most of the period of growth, disks inoculated with E. coli again showed the least uptake of

water and those infected with A. tumefaciens the most. Between three and four weeks however the values of the ratio for all treatments were very similar.

3.20 Comparison of the Dry Weight to Fresh Weight Ratio for Disks Grown under the Three Conditions.

In all light conditions, disks inoculated with A. tumefaciens showed most water uptake, and this was greatest in low light. In high light, uninfected and E. coli-inoculated disks had a similar dry weight to fresh weight ratio. However, in low light and the dark, disks inoculated with E. coli had the lowest fresh weights, and the highest dry weight to fresh weight ratio, showing that the little growth occurring was more by cell division than enlargement. This observation was confirmed by histological studies of similar disks (see Chapter 9).

3.21 Conclusions.

Decapitated seedlings inoculated with E. coli and A. tumefaciens reacted in different ways, contrary to Philipson and Sheat's report (1963). No callus comparable in size or character to crown gall was induced in E. coli-inoculated hypocotyls. The callus formed in such hypocotyls appeared to be the result of an intensified wound response.

With hypocotyl disks, it was clearly seen that E. coli-infected disks proliferated in quite a different manner from those inoculated with A. tumefaciens. In addition, an interesting differential stimulation of E. coli-infected disks in different light intensities was noted. It was apparent that E. coli-

infected disks were stimulated to grow more than uninfected controls in the high light, while in low light and the dark, the infected disks were inhibited. It would appear that some substance associated with the bacteria was active which was different in character from the stimulus produced by A. tumefaciens.

EXPERIMENT 6. EFFECTS OF DIFFERENT FORMS OF INOCULUM ON THE RESPONSE OF DECAPITATED HYPOCOTYLS

Because of discrepancies between reactions of decapitated hypocotyls in previous experiments, another attempt was made to assess the effects of bacteria when applied to these tissues. The seedlings used had been obtained from a trial in which different methods of seed sterilization were evaluated (see Chapter 2).

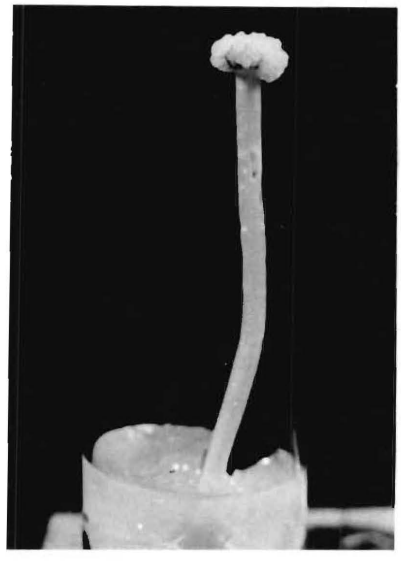
In a number of reports describing inoculation with A. tumefaciens the inoculum was obtained by flooding a culture on an agar slope with sterile water (de Ropp 1951c).

An experiment was prepared to determine whether better results could be obtained by using this type of inoculum rather than a culture of the bacteria grown in nutrient broth.

3.22 Experimental Details.

Sterile seedlings were decapitated and inoculated with a loop-ful of either E. coli or A. tumefaciens suspended in sterile water or nutrient broth cultures of these bacteria. Some hypocotyls were inoculated with sterile broth or water. All hypocotyls were grown in the dark after inoculation. Responses were recorded 16 to 19 days after treatment.

a) Hypocotyl inoculated with a suspension of E.coli in distilled water.



b) Hypocotyl inoculated with a broth culture of A.tumefaciens.

c) Hypocotyl inoculated with a suspension of A.tumefaciens.

FIGURE 3.9 DECAPITATED HYPOCOTYLS INOCULATED WITH DIFFERENT BACTERIA (Mag. x1.13)

3.23 Results and Discussion.

The reactions of the hypocotyls are tabulated in a similar way to those of preceding experiments (Table 3.15).

TABLE 3.15. REACTIONS OF HYPOCOTYLS TO INOCULATION

BACTERIAL TREATMENT	MEDIUM	NUMBER OF HYPOCOTYLS						
		POSITIVE RESPONSE				NO REACTION	DEAD	TOTAL
		+	++	+++	++++			
E. coli	Broth	3	2	1	1	1	8	16
	Suspension		4	7	1	4	6	22
A. tumefaciens								
	Broth				1	1	5	7
	Suspension	2	1	4				7
Control	Broth	2	1			2	8	13
	Suspension	2				6	7	15

There was no significant difference between the amounts of callusing when E. coli was applied in the two different ways although the number of hypocotyls forming large calluses was increased slightly when the bacteria were applied in a suspension with sterile water. (For details of significance tests see Appendix I). The largest callus induced by inoculation with E. coli in all the experiments was formed when the bacteria were applied in this suspension (Figure 3.9a).

Many calluses were formed when hypocotyls were inoculated with A. tumefaciens in a suspension with sterile water, but when nutrient

broth cultures were used, most of the hypocotyls either died or did not react. The largest crown gall, however, was formed on a hypocotyl which had been inoculated with a broth culture of the bacteria (Figure 3.9b). In one of the hypocotyls inoculated with the A. tumefaciens suspension, a secondary gall was formed below the cut surface and there was very little callusing at the surface where the bacteria were applied (Figure 3.9c).

It was decided to continue the use of the E. coli broth cultures as significantly different results were not obtained by the two methods.

3.24 Discussion of the Preliminary Experiments.

In the experiments using decapitated seedlings it became evident that no constancy of results could be obtained with this method. In Experiment 1 hypocotyls grown in the dark responded to inoculation with either E. coli or sterile nutrient broth, by the production of many calluses, but in the light most hypocotyls died. Seedlings in the second experiment were only grown in the dark, and those inoculated with E. coli formed more large calluses than uninfected hypocotyls. A further comparison was made between decapitated hypocotyls grown in light and dark in Experiment 4, when it was found that more callusing resulted when infected hypocotyls were grown in the light than in the dark, the reverse of observations in the first experiment. Finally, a comparison was made among inoculated hypocotyls grown in three light intensities. In this case, most reacted by swelling below the cut surface, and

only occasionally was any callus produced.

In addition to the inconsistency in reaction of the decapitated hypocotyls grown in different light conditions, there was the problem that the normal wound response of uninfected hypocotyls was in some cases indistinguishable from the response of those inoculated with E. coli. Hypocotyls inoculated with A. tumefaciens, however, showed a distinct response at the site of inoculation which was easily distinguished from the normal wound response of uninfected seedlings. Although there were no definite differences between light and dark-grown hypocotyls, either uninfected or inoculated with E. coli, it appeared that hypocotyls inoculated with A. tumefaciens did not proliferate as much when grown in the dark as in the light.

In contrast with decapitated seedlings, hypocotyl disks showed more constant and readily detectable differences between the bacterial treatments. While the proliferation induced by A. tumefaciens was similar to that found in decapitated hypocotyls, disks inoculated with E. coli proliferated in a distinctive manner when grown in high light, and were easily distinguishable from uninfected disks. Although no constant differences were found between decapitated seedlings inoculated with E. coli and grown in light or dark, there were distinct differences in the reactions of infected disks grown under these conditions. Those grown in the high light showed a large amount of proliferation from the lower surfaces and formed long roots while those in the low light or darkness grew only a little and formed short roots.

The reactions of disks inoculated with A. tumefaciens were similar in all light conditions, although dark-grown disks were smaller. With this bacterial treatment proliferation was induced at the upper surface of the hypocotyl disks and a few short roots formed. In both experiments with hypocotyl disks those inoculated with A. tumefaciens showed the highest water uptake in all conditions, while in the low light and dark, E. coli-inoculated disks showed the least water uptake. Values for uninfected disks were intermediate between these, and in the high light, uninfected and E. coli-inoculated disks showed similar amounts of cell division and water uptake.

The experiments with both decapitated seedlings and hypocotyl disks, have shown that infection with E. coli induces a reaction essentially different from that produced by A. tumefaciens. The few tests with B. megaterium also showed that its effect on tissues was different from that of E. coli.

It is apparent that E. coli induces stimulation of sunflower tissue by the formation of some active substance either in the culture medium or in conjunction with the plant tissues. The influence of this substance on tissues is quite different from that found for B. megaterium, and is also quite distinct from the effects of A. tumefaciens, because of its inhibiting properties on tissues grown in low light or darkness.

CHAPTER FOUR

INOCULATION OF OLDER SUNFLOWER PLANTS ALONE OR IN CONJUNCTION WITH IAA

The experiments described so far were concerned with the reactions of young decapitated sunflowers and hypocotyl disks to inoculation with Escherichia coli. The response to this bacterium of older plants not grown under controlled conditions was also investigated. Philipson and Sheat (1963) reported that no callus was induced when two to three week old sunflower seedlings, grown in glasshouse conditions, were inoculated with E. coli. They attributed this to rapid wound healing in relatively dry air. However, local stem swellings occurred when inoculated plants of the same age were grown in a humid atmosphere.

EXPERIMENT 7a. EFFECTS OF INOCULATING THE HYPOCOTYL OR FIRST INTERNODE OF INTACT SUNFLOWERS

Because of the analogies made by Philipson and Sheat (1963) between E. coli-induced callus and crown galls on decapitated seedlings, a comparison was made between the responses of intact plants grown under glasshouse conditions and infected with E. coli and A. tumefaciens. This was carried out shortly after the start of experimental work and refinements of technique mentioned in Chapter 3 had not been made at this time.

4.1 Experimental Details.

Sunflower plants, grown in a glasshouse for four weeks until

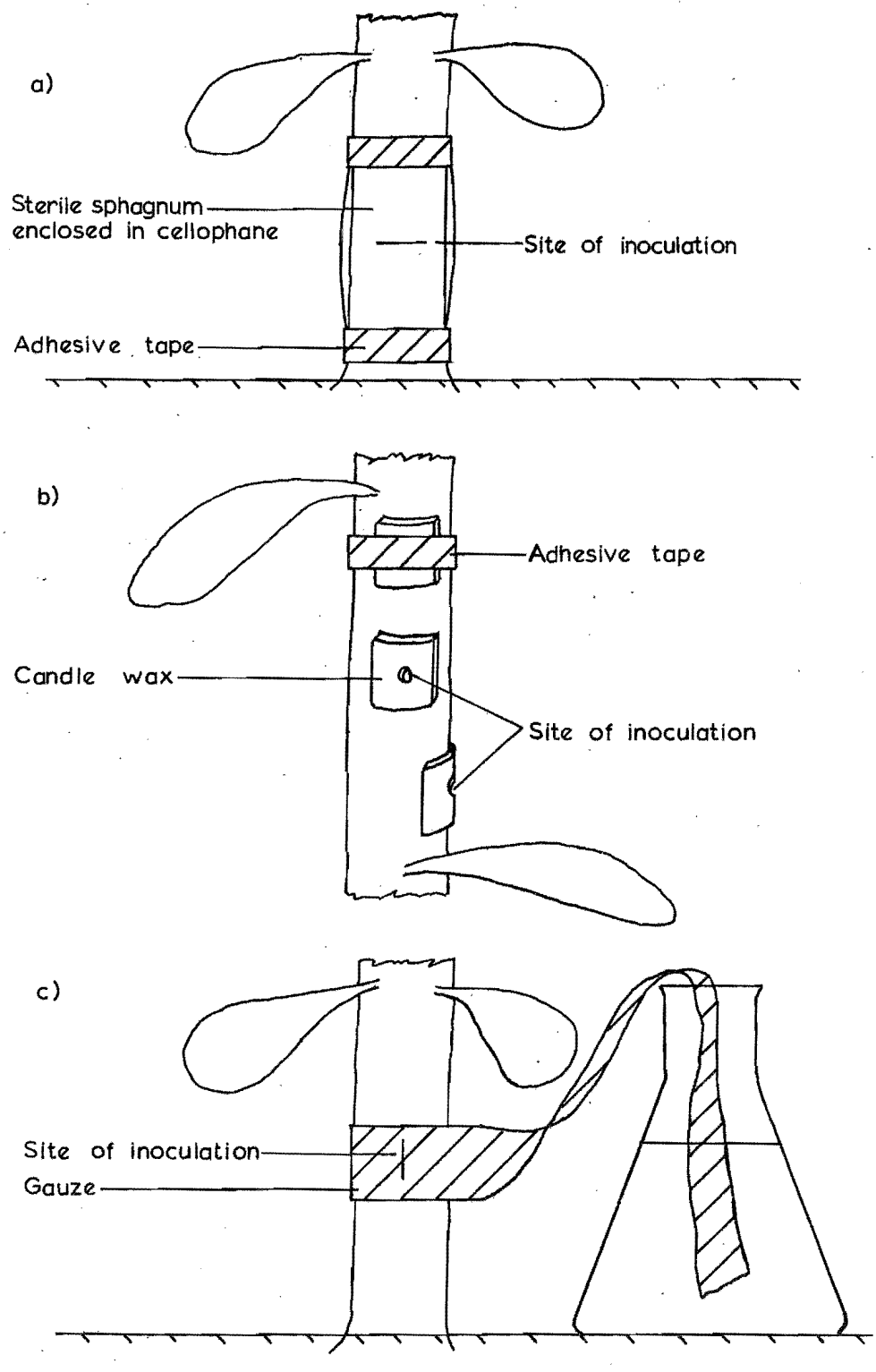


FIGURE 4.1 POSITION OF INOCULATION AND METHOD OF COVERING THE WOUND

they were 30 to 40 cm tall, were inoculated by a horizontal cut in the hypocotyl with a sterile scalpel which had previously been immersed in the particular inoculum required. Sterile nutrient broth or 48-hour cultures of E. coli or A. tumefaciens in nutrient broth were used. In both bacterial cultures, strains had been selected for virulence by several passages through decapitated sunflower seedlings as described in Chapter 3. Moist sphagnum moss, sterilized by autoclaving for one hour at 120°C on two consecutive days, was wrapped around the wound, and was held in position by sterile cellophane taped to the hypocotyl at both ends to exclude the passage of micro-organisms, and to maintain a moist environment in the vicinity of the wound (Figure 4.1a). It was thought that a moist environment would be more suited to the formation of E. coli calluses, since these were formed more readily on decapitated hypocotyls grown in the dark, than in the light where the atmosphere was drier. In addition Philipson and Sheat found that swelling followed inoculation with E. coli when the plants were held in a damp atmosphere, although in their case sterile conditions were maintained in a closed vessel. Of the twelve sunflowers treated in this way in the present experiment, six were inoculated with a culture of E. coli, two with A. tumefaciens and four with sterile nutrient broth. A further six sunflowers six weeks old were inoculated by a vertical cut in the first internode above the hypocotyl because the hypocotyls of these older plants had split as a result of rapid growth. The wound was again maintained in a moist environment with sphagnum. Five of these sunflowers were inoculated with a culture of E. coli

TABLE 4.1 REACTION OF ENTIRE SUNFLOWER PLANTS INOCULATED IN THE HYPOCOTYL OR FIRST INTERNODE

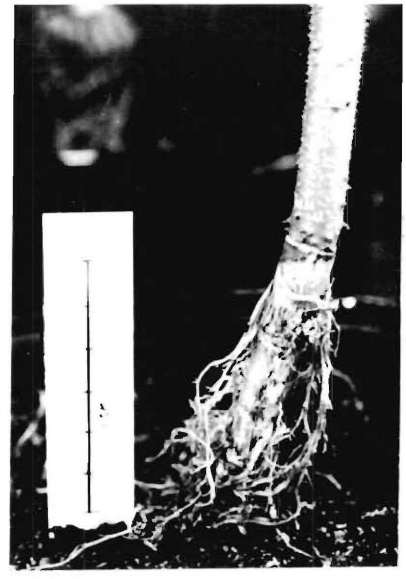
TREATMENT	INOCULUM	NUMBER OF PLANTS								
		PROLIFERATION			TOTAL	ROOT FORMATION				TOTAL
		-	+++	++++		-	+	++	+++ ++++	
Horizontal stab in the hypocotyl	Nutrient broth	4			4		3	1		4
	E. coli (S_5)	6			6		1	2	3	6
	A. tumefaciens (S_5)		1	1	2			2		2
Vertical stab in first internode	Nutrient broth	1			1		1			1
	E. coli (S_5)	5			5	2	1	2		5

Proliferation

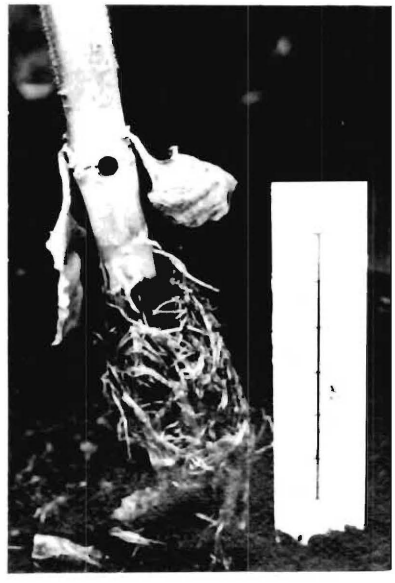
- none
+++ large gall
++++ very large gall

Root formation

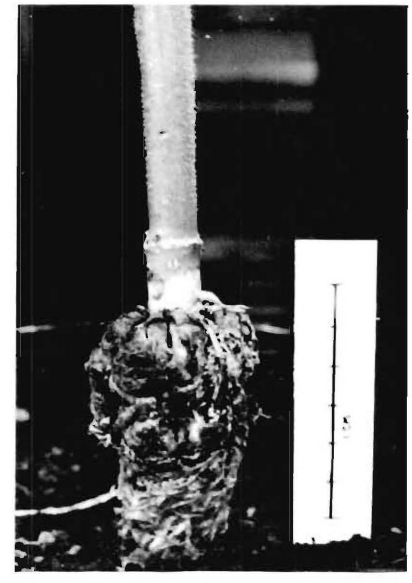
+ very few formed
++
+++ ↓
++++ hypocotyl completely enveloped in roots.



a) Hypocotyl inoculated with sterile nutrient broth.



b) Hypocotyl inoculated with E.coli.



c) Hypocotyl inoculated with A.tumefaciens.

FIGURE 4.2 HYPOCOTYLS OF INTACT PLANTS TWO MONTHS AFTER INOCULATION

and one with sterile nutrient broth.

Results were recorded in plants inoculated in the hypocotyl with a horizontal cut seven and a half weeks after inoculation, and in those treated at the first internode after five and a half weeks (Table 4.1).

4.2 Results and Discussion.

All of the sunflowers inoculated in the hypocotyl produced roots in this region and only with A. tumefaciens-infection were any galls formed. These galls were very large and rather woody, extending at least half way around the diameter of the hypocotyl and vertically for about 2 cm on either side of the wound (Figure 4.2c). In hypocotyls inoculated with sterile nutrient medium only a few roots were produced (Figure 4.2a). Of the hypocotyls inoculated with E. coli one had produced fewer roots than the controls, two had produced as many as the controls and three showed more abundant root formation (Figure 4.2b). In both E. coli-inoculated and uninfected plants the wound healed with little, if any, callus formation. Five of the plants infected with E. coli retained their cotyledons, as did two A. tumefaciens-inoculated and two uninfected plants.

The older plants inoculated at the first internode showed very little response to this treatment. In all cases the wound healed with the formation of a little callus. Four of the five plants inoculated with E. coli produced a few roots from the stem but these were much fewer in number than those formed on the uninfected plant.

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This work showed that whole plants inoculated with A. tumefaciens reacted to the bacteria in a different manner from plants infected with E. coli. Only small numbers of plants were used in this experiment, but the results suggested that E. coli influenced the plant by causing an increased amount of root formation.

Appler (1951) found that when crown galls were formed on sunflower plants, or when indole acetic acid (IAA)-lanolin paste was applied to the decapitated stems, the lifespan of the cotyledons was increased. Although only small numbers of plants were used in the present work those inoculated with A. tumefaciens showed no increased retention of the cotyledons as compared with uninfected plants while the numbers of cotyledons retained were greatly increased with E. coli infection. This effect observed by Appler in connection with A. tumefaciens infection or the presence of auxin, may have been partially due to the age of the sunflower plants when decapitated and inoculated, since La Rue and MacNeill (1933) reported that when the plumules of various plants were removed the cotyledons lived much longer. The plants used in the present experiment were relatively old and the stem apex was not removed. No definite conclusions can thus be made from the character of retention of the cotyledons, although the increased numbers in E. coli-infected plants as compared with uninfected plants might be attributed to changed growth substance metabolism.

The increased root formation from the hypocotyls of plants

infected with E. coli, together with the retention of the cotyledons, suggested that a growth substance similar to IAA might be produced under the influence of the bacterial treatment.

EXPERIMENT 7b. EFFECTS OF INOCULATING THE STEM OF INTACT SUNFLOWERS

Three months later a further experiment was prepared to induce callus formation in whole plants infected with E. coli, using different methods of inoculation and maintenance of a moist atmosphere.

4.3 Experimental Details.

Sunflowers one month old were inoculated by puncturing the stem with a hypodermic syringe containing the inoculum, either E. coli, A. tumefaciens, or sterile nutrient broth. The wound was surrounded by candle wax which was then covered with polythene adhesive tape (Figure 4.1b). This provided a moist chamber about the wound. Difficulty was experienced in attaching wax to the stem, so that in some cases the wound was kept moist by a layer of lanolin. Three inoculations were made at different internodes of the plant, nine plants being inoculated with E. coli, four with sterile broth and three with A. tumefaciens. The wax and adhesive tape were removed after 15 days so that growth near the wound could be observed.

4.4 Results and Discussion.

The plants were grown in the winter and appeared to be infected with a virus disease since there were necrotic lesions on many of

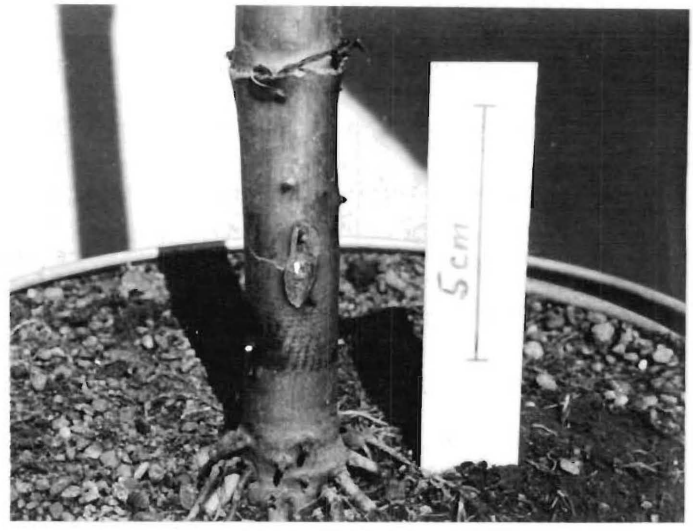
the leaves. In plants inoculated with either E. coli or sterile broth, occasional swelling about the wound occurred, but in general, no reaction was observed and no roots developed. In all cases, plants inoculated with A. tumefaciens developed galls, the proliferation of which was confined to the cavity enclosed by wax.

EXPERIMENT 7c. EFFECTS OF INOCULATING THE HYPOCOTYL OF INTACT SUNFLOWERS

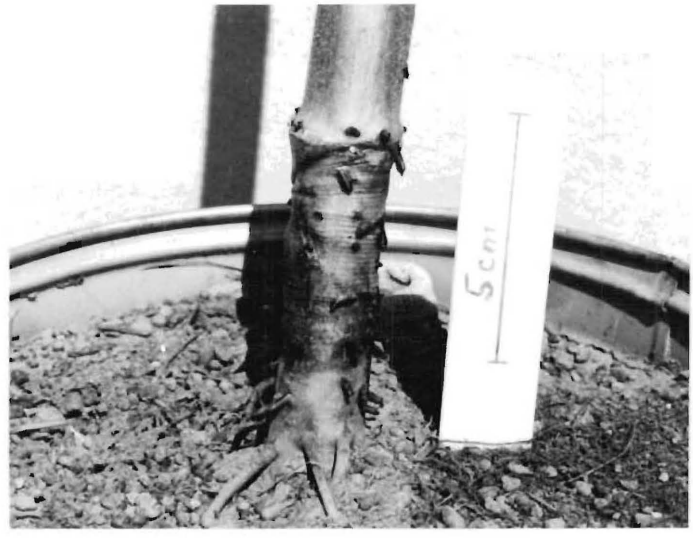
Eighteen months later a further attempt was made to induce callus formation and rooting in the hypocotyls of intact sunflowers grown in the glasshouse and inoculated with E. coli.

4.5 Experimental Details.

Sunflower plants five weeks old with two or more often three pairs of expanded leaves, were inoculated in the hypocotyl by a vertical stab with a scalpel coated in the inoculum. To maintain a moist atmosphere in the vicinity of the wound the hypocotyl was wrapped in gauze, the ends of which rested in a flask containing distilled water (Figure 4.1c). In this way the gauze about the wound was always damp. The flasks containing distilled water were refilled every day, because evaporation was very rapid. This method of keeping the wound moist was used by Riker (1926) for plants inoculated with A. tumefaciens. Ten plants were inoculated with a culture of E. coli in synthetic medium and ten with sterile synthetic medium. The gauze was removed and the hypocotyls examined four weeks after inoculation.



a) Hypocotyl inoculated with sterile synthetic medium.



b) Hypocotyl inoculated with E.coli.

FIGURE 4.3 HYPOCOTYLS OF INTACT PLANTS SIX WEEKS AFTER INOCULATION

4.6 Results and Discussion.

With both treatments the wounds in the hypocotyls healed with little or no proliferation of the tissues. Of the ten uninfected plants, five produced no roots while small root primordia or a few short roots were present in the other five. In two hypocotyls inoculated with E. coli there were no roots, while one had small root primordia and the remaining seven had formed many roots up to 1.5 cm long (Figure 4.3, Table 4.2).

TABLE 4.2 ROOT FORMATION IN INOCULATED HYPOCOTYLS OF INTACT PLANTS

TREATMENT	NUMBER OF PLANTS RESPONDING		
	No roots	Primordia or small roots.	Large roots
Synthetic Medium	5	5	0
E.coli	2	1	7

A comparison of root formation in the two treatments by a χ^2 test showed that plants inoculated with E. coli were stimulated to produce a significantly greater (at the 1% level) number of roots than the uninfected plants (Appendix II).

These results confirmed the findings of the first experiment of this series, showing that although no callus formation occurred in whole sunflower plants inoculated with E. coli when the wound was kept moist, root initiation near the wound was stimulated. It seemed likely that the stimulus to root formation was of an auxinic nature.

EXPERIMENT 8. EFFECTS OF IAA ON DECAPITATED INOCULATED SUNFLOWERS

The results of the previous experiments with intact sunflowers led to a further experiment being prepared to determine the effects of IAA on bacterial-inoculated plants grown in the glasshouse. Braun and Laskaris (1942) and Thomas and Riker (1948) showed that tumour formation in the presence of attenuated strains of A. tumefaciens could be induced if growth substances were added to the cut surface of decapitated plants after their inoculation. From this they inferred that the bacteria could incite gall formation but that a growth substance was required before the initiated gall could proliferate. It seemed possible that E. coli might act in a similar way to an attenuated strain of A. tumefaciens and that the bacteria and growth substances might act synergistically in the formation of callus. Some evidence supporting this had already been obtained from an experiment described in Chapter 5 with inoculated hypocotyl disks grown on media containing IAA.

4.7 Experimental Details.

Sunflower plants five weeks old were decapitated 3 to 4 cm above the third node and punctured 0.5 cm and 2 cm below the cut surface with a sterilized needle which had been dipped in the required inoculum. The cut surface of the stem was treated either immediately the plants had been inoculated, or after four days, with different concentrations of IAA in lanolin paste. The delay in the application of the auxin was to determine whether the IAA could act in conjunction with the bacteria in initiating the galls or whether the bacteria alone could initiate galls and the IAA stimulate their proliferation following initiation, as in crown

gall. The method used was described by Braun and Laskaris (1942). Plants were inoculated with a 24-hour culture of E. coli in synthetic medium, a culture of A. tumefaciens in nutrient broth or with sterile synthetic medium. A chemically defined synthetic medium was used for the culture of E. coli because it supported the growth of this bacterium and did not contain tryptophane. The significance of this is discussed following the results of this experiment. The IAA was applied in a lanolin paste in the concentrations used by Braun and Laskaris, namely 2.5%, 2%, 1.5% and 1%, and a control with no IAA present in the lanolin was also used. The IAA-lanolin paste was prepared by weighing the required amount of IAA and dissolving it in 1 to 2 cc of absolute alcohol. This solution was added to the correct quantity of hydrous lanolin and the two were thoroughly mixed.

Five replicates of each combination of bacterial and IAA treatments were prepared, except when A. tumefaciens was used. The IAA-lanolin paste was applied to two plants of each treatment immediately after inoculation, and in the remaining three plants it was applied four days later. The experimental design was as follows:-

IAA(%)		INOCULUM								
		Sterile synthetic medium			E. coli			A. tumefaciens		
		Reaction		Callus diam. -cm.	Reaction		Callus diam. -cm.	Reaction		Callus diam. -cm.
		11 days	38 days		11 days	38 days		11 days	38 days	
Nil	a)	-	-	0.85	-	Died	-	SS	+1	4.96
		-	-	1.13	-	-	0.96			
	b)	-	-	1.11	-	-	0.94	SS	++	3.02
		-	-	1.00	-	-	0.91			
		-	-	0.83	-	-	1.00			
1	a)	++	++	2.78	++SS	++	2.47			
		SS	++	2.31	+S	++	3.61			
	b)	S	++	1.77	-	++S	2.2	SS	++	4.18
		S	++	1.92	-	++	2.69			
		S	++	2.20	-	++	1.72			
1.5	a)	+	++	1.95	S	+S	1.78			
		+	++	2.30	SS	++	3.69			
	b)	++	++	2.33	S	++	1.50	SS	++	5.24
		++	+Died	1.76	S	++	1.93			
		++	++	2.60	-	++	2.25			
2	a)	SS	SS	1.46	++SS	++	2.76			
		SS	++	2.86	SS	++	3.48			
	b)	+	++	2.45	SS	+S	2.81	++SS	++	4.67
		++	++	2.37	S	+S	1.67			
		S	++	2.48	S	++	2.04			
2.5	a)	SS	++SS	2.97	SS	++	2.43			
		SS	++SS	2.32	SS	++	2.57			
	b)	S	++	2.70	SS	+	1.89	S	+	3.12
		SS	++	2.10	SS	+	1.56			
		SS	++	1.74	SS	+	2.45			

- No callusing or swelling. + Moderate callusing. ++ Large callusing. S Moderate swelling. SS Large swelling.

TABLE 4.4 LATERAL BUD FORMATION AND EPINASTY OF LEAVES IN TREATED DECAPITATED SUNFLOWERS

IAA(%)		INOCULUM								
		STERILE SYNTHETIC MEDIUM			E. COLI			A. TUMEFACIENS		
		Lateral Buds		Epinasty	Lateral buds		Epinasty	Lateral buds		Epinasty
		11 days	21 days	11 days	11 days	21 days	11 days	11 days	21 days	11 days
nil	a)	-	2 large branches	-	+	2 large branches	+	+	1 large branch	+
		++		-	+		-			
	b)	+		-	++		+	++		-
		++		-	++		-			
		++		-	+		-			
1	a)	++	2 medium branches	+	+		-			
		+	2 large branches	+	+	1 medium branch	-			
	b)	++		-	+		-	++	2 large branches	-
		++		-	++		-			
		++		-	++		-			
1.5	a)	+	1 very small bud	+	-	1 small branch	-			
		+		-	-		+			
	b)	++		+	++		+	++	2 large branches	+
		++		-	+		-			
		+		-	+		+			
2	a)	+	2 small branches	+	++	2 large branches	-			
		+	None	+	-		+			
	b)	+		-	++		+	++	2 large branches	+
		+		-	+		-			
		++		-	+		-			
2.5	a)	++	2 medium branches	+	++	2 large branches	+			
		-	None	+	++		+			
	b)	-		+	++		-	++	2 small stunted branches	-
		++		-	-		+			
		++		-	-		+			

Lateral buds - absent

Epinasty - absent

Concentration of IAA		Sterile synthetic medium	NUMBER OF PLANTS INOCULUM ·	
			E. coli	A. tumefaciens
No IAA	a	2	2	1
	b	3	3	1
1% IAA	a	2	2	-
	b	3	3	1
1.5% IAA	a	2	2	-
	b	3	3	1
2% IAA	a	2	2	-
	b	3	3	1
2.5% IAA	a	2	2	-
	b	3	3	1

a IAA applied immediately following decapitation
b IAA applied four days after decapitation and inoculation

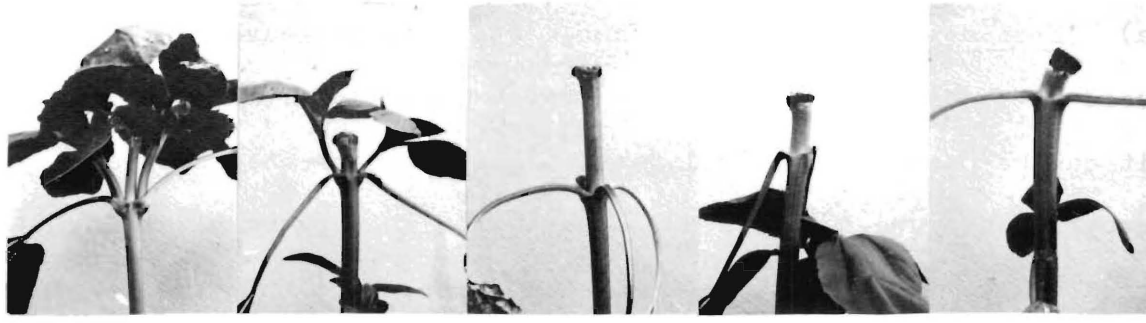
Only six plants were inoculated with A. tumefaciens and of these four were treated with IAA four days later, and lanolin was applied immediately after inoculation to one plant, and after four days to the remaining plant. In all cases the IAA-lanolin paste was renewed at seven day intervals until three applications had been made.

The reactions of the plants to the treatments were recorded over two months.

4.8 Results.

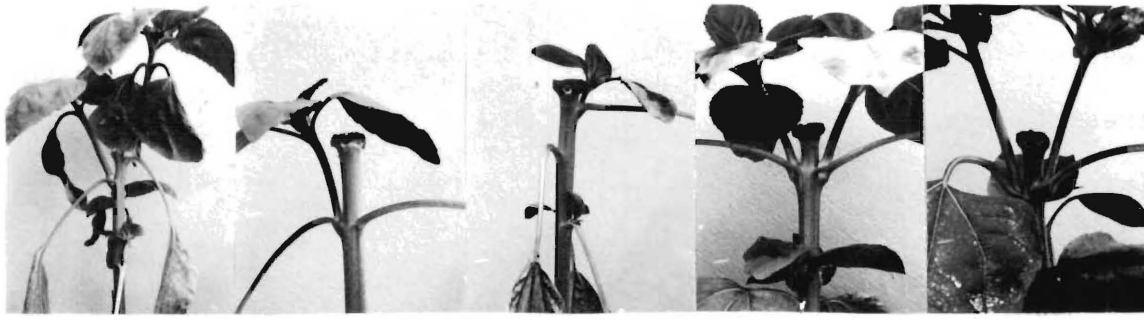
In no case was proliferation induced at the sites of inoculation of plants treated with sterile synthetic medium or E. coli. Where the stems had been punctured all plants inoculated with A. tumefaciens produced galls which became large and woody after two months.

The responses of the plants to the growth substances and bacteria have been summarized in Tables 4.3 and 4.4.



No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

Plants inoculated with sterile synthetic medium.



No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

Plants inoculated with E.coli.

No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

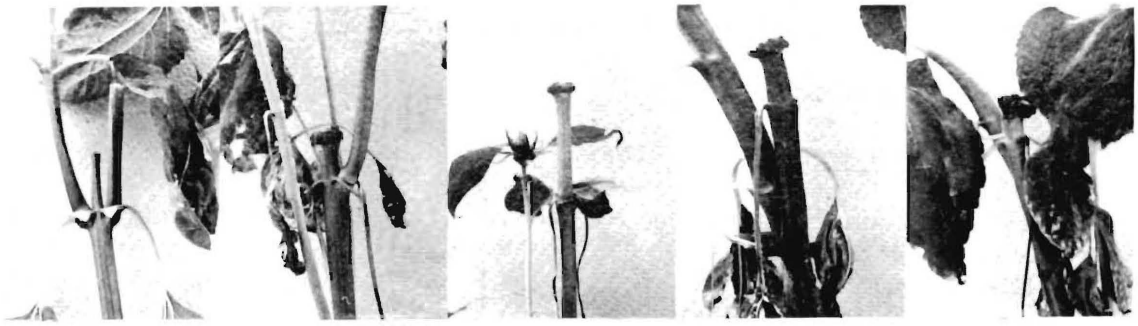
Plants inoculated with A.tumefaciens.

FIGURE 4.4 EPINASTIC RESPONSE, LATERAL BUD FORMATION AND CALLUSING IN TREATED SUNFLOWERS 21 DAYS AFTER INOCULATION

a) Epinastic Response. Although epinasty of leaves cannot be used as a quantitative test for growth substances (Audus 1959) it can be used qualitatively as an indication of the changes in growth substance metabolism. Apart from uninfected plants treated only with lanolin, and E. coli-inoculated plants treated with 1% IAA-lanolin paste, some plants in all treatments showed an epinastic response of the leaves eleven days after inoculation. After three weeks, all the uninfected plants to which varying concentrations of IAA had been applied showed a marked epinastic response (Figure 4.4). Plants inoculated with E. coli but not treated with IAA showed a large epinastic response. E. coli-infected plants treated with 1% and 1.5% IAA also showed some response, although this was not as great as in uninfected plants treated with the same concentrations of IAA. Little epinasty occurred in infected plants treated with 2% and 2.5% of IAA-lanolin paste (Figure 4.4). In all plants inoculated with A. tumefaciens, irrespective of the amount of IAA applied to the cut surface, the petioles curved markedly, showing the greatest epinastic response of all plants treated. The differences in the amount of epinasty of the leaves observed in the variously treated plants were probably due to the inherent variability of the plant material.

b) Axillary Bud Formation. Thimann and Skoog (1934) reported early in the experimental work with auxins that the addition of IAA to a decapitated stem could replace the function of the terminal bud in maintaining apical dominance, and thus inhibit

the growth of lateral buds. In the present work, most plants formed axillary buds but their continued growth was dependent on the particular treatment of the plant. Again, as with leaf epinasty, variations occurred within the treatments. After three weeks of growth large axillary branches were present in uninfected plants to which only lanolin had been applied, while in the uninfected plants treated with IAA, the growth of the buds was slower and they remained small with higher concentrations of auxin. With E. coli inoculation, plants not treated with IAA produced large axillary branches comparable in size to the uninfected controls. Plants treated with 1% and 1.5% of IAA in lanolin paste formed fewer buds which grew less than plants without added auxin. This bud formation was similar to that in uninfected plants which had been treated with the same concentration of IAA. With IAA concentrations of 2% and 2.5% E. coli-infected plants produced very large axillary branches (Figure 4.4). Appler (1951) reported terminal crown gall tumours to have the same effect as applied auxin in maintaining apical dominance, so that growth of axillary buds is inhibited. In the present experiment however the plants inoculated with A. tumefaciens produced large axillary buds in all treatments when auxin was also added, except when the IAA was at a concentration of 2.5%. Small, very stunted axillary buds were formed in this case. The uninfected plants therefore reacted in the expected way by a reduction of lateral bud formation with increasing IAA concentration, while bud formation in the infected



No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

Plants inoculated with sterile synthetic medium.

No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

Plants inoculated with E.coli.

No IAA 1% IAA 1.5% IAA 2% IAA 2.5% IAA

Plants inoculated with A.tumefaciens.

FIGURE 4.5 PRODUCTION OF CALLUS IN TREATED SUNFLOWERS 54 DAYS AFTER INOCULATION

plants was affected both by the concentration of IAA and the bacterial treatment.

c) Callus Formation. In uninfected and E. coli-inoculated plants, the cut surface did not proliferate when it was treated with lanolin alone. Eleven days after inoculation, all plants to which IAA had been immediately applied showed some signs of swelling near the cut surface, while uninfected plants treated with 1% and 1.5% IAA had proliferated. There was not as much swelling in E. coli-inoculated plants when the auxin paste was not added for four days. Three weeks after inoculation all plants treated with IAA showed some proliferation of the cut surface. The reaction was again less marked in plants which received IAA applications four days after they had been inoculated, particularly at concentrations of 2% and 2.5% of IAA. Little callusing occurred at the cut surface of plants inoculated with A. tumefaciens although in one case, where a plant was treated with 2% IAA-lanolin paste, proliferation did occur. Plants inoculated with E. coli and treated with concentrations of IAA at the 1.5%, 2% and 2.5% levels immediately after inoculation formed larger calluses than uninfected plants receiving comparable amounts of IAA.

After the plants had been inoculated for nearly two months many of the axillary shoots bore flowers, and vegetative growth had ceased. An increase in the callusing at the cut stem surface had occurred since that recorded when the plants were three weeks old (Figure 4.5). Measurements of the diameters

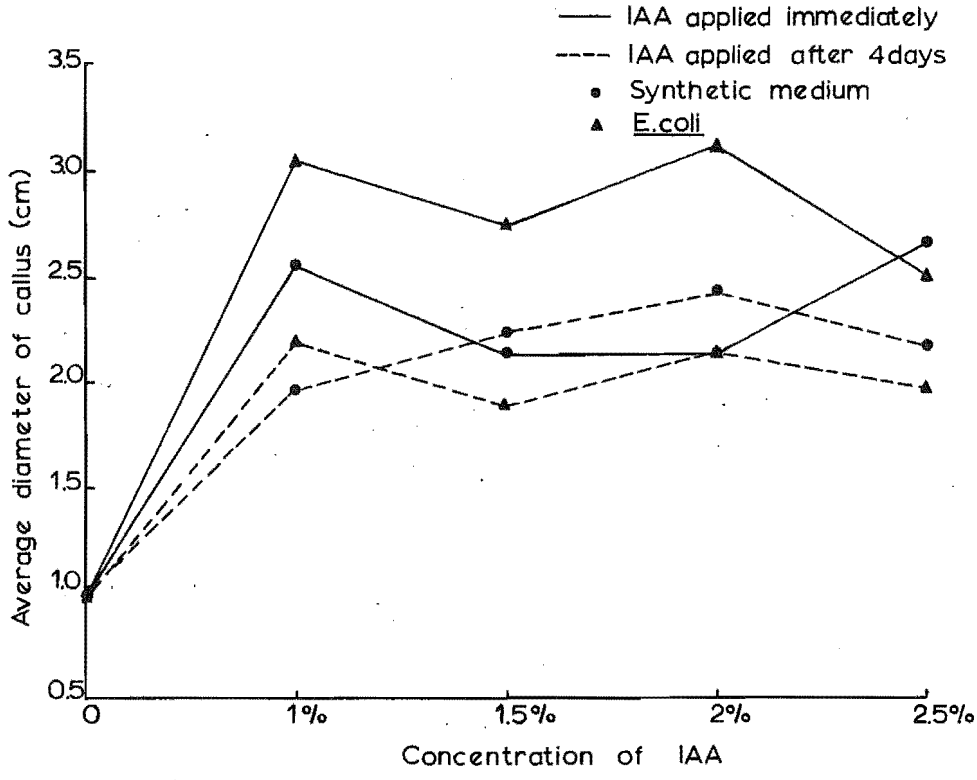


FIGURE 4.6 AVERAGE DIAMETER OF CALLUSES AFTER 54 DAYS WITH IAA APPLIED IMMEDIATELY AND 4 DAYS AFTER INOCULATION

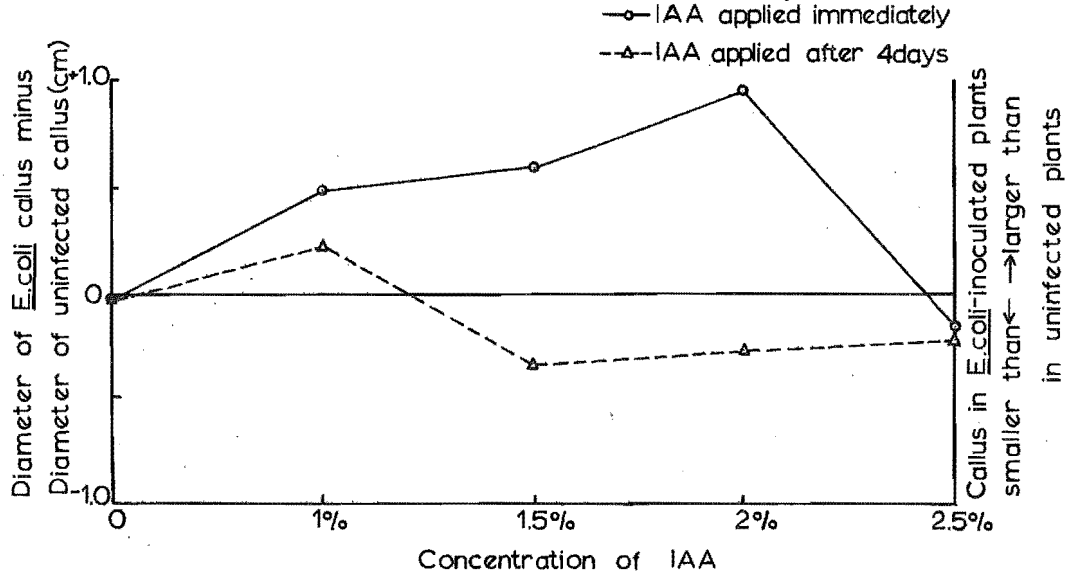


FIGURE 4.7 DIFFERENCE IN DIAMETER OF CALLUS IN E.COLI-INOCULATED AND UNINFECTED PLANTS AFTER 54 DAYS

of the calluses were made (Table 4.3). The largest calluses were obtained in plants inoculated with E. coli to which the IAA-lanolin paste had been applied immediately after inoculation (Figures 4.6 and 4.7).

When the plants were treated with lanolin which did not contain auxin, E. coli-infected stems were slightly smaller than uninfected stems and there was no callusing. With auxin added immediately in concentrations from 1% to 2% increasingly larger calluses were formed in plants inoculated with E. coli, as compared with the uninfected plants, while at concentrations of 2.5% IAA, E. coli-infected plants produced slightly smaller calluses than their uninfected counterparts (Figure 4.7). In only two cases, which occurred in uninfected plants to which 1.5% and 2% IAA had been added, the average diameters of the calluses were smaller in plants which had been treated with auxin immediately than when the IAA was added four days later. For all other plants, either uninfected or inoculated with E. coli, calluses were larger in plants treated with auxin immediately following decapitation (Figure 4.6).

All plants infected with A. tumefaciens were treated with varying concentrations of IAA four days after inoculation since Braun and Laskaris (1942) showed that the auxin stimulated proliferation of the gall cells after their initiation. The size of the crown galls formed at the sites of inoculation increased with increasing IAA concentration from 1% to 1.5%. Plants treated with 2% IAA formed smaller galls than those treated with

1.5% IAA. Application of 2.5% IAA apparently had no stimulating effect on the size of the galls compared with those formed when plain lanolin was added to the cut surface. The two A. tumefaciens-infected plants treated with plain lanolin produced galls of different sizes, the larger one being formed when the lanolin was added immediately after inoculation.

4.9 Discussion.

The presence of E. coli in a wound close to a cut stem surface to which IAA-lanolin paste was applied following inoculation stimulated the production of callus at the cut surface. When IAA-lanolin paste was applied four days after inoculation, this stimulation was not apparent and in fact inhibition of callusing occurred in comparison with uninfected plants (Figure 4.7). In the experiments described by Braun and Laskaris (1942) stimulation occurred when the auxin was added to the plants four days after inoculation showing that the induction of tumours was a separate process from their continued growth. In this respect, the action of E. coli in conjunction with IAA in sunflower plants was quite different from that of A. tumefaciens. Besides the enhanced effect on callusing from the cut surface when this auxin was applied immediately after inoculation, no proliferation was stimulated at the sites of inoculation. To produce this effect it seemed likely that the bacteria were stimulating growth by the formation of auxin in the presence of the plant tissue.

It is well known that certain species of bacteria and fungi

are able to produce IAA in culture. This was first demonstrated by Hopkins and Cole (1903) for cultures of E. coli. When the growth-promoting properties of A. tumefaciens were first investigated it was suggested that the bacteria were synthesizing IAA, and in fact this proved to be correct (Berthelot and Amoureaux 1938), but it was found that the quantities of IAA formed were not sufficient to produce the lasting effects induced by the bacteria in the plant tissues (Riker, Henry and Duggar, 1941, de Ropp 1951b).

Several examples correlating the pathogenicity of fungi with their ability to produce IAA have been recorded (Wolf 1952, Berducou 1952). Mycorrhizal fungi have also been found to synthesize indole compounds, including IAA (Ulrich 1960, Moser 1959). However, not all bacteria and fungi producing IAA are plant pathogens or symbionts (Roberts and Roberts 1939).

A full list of known producers of IAA has been given by Fallot (1964). In most cases it has been shown that these fungi and bacteria require the presence of tryptophane in the culture medium in order to produce IAA. Tryptophane is a precursor of IAA and is easily converted to the auxin by autoclaving (Burkholder 1939, Fallot 1964).

Although E. coli was the first bacterium shown to produce IAA, to the writer's knowledge no results have been published on the production of IAA in cultures of E. coli containing only inorganic nitrogen. Fallot (1964) reported that Burkholder (1939) found IAA to be produced by E. coli in media without

tryptophane. This appears to be an error however as reference to the original paper showed that only Aerobacter aerogenes was tested on a tryptophane-free medium.

After the initial experiments in the present work, all cultures of E. coli were made in a synthetic, chemically defined medium, containing ammonium di-hydrogen orthophosphate as the only source of nitrogen. No IAA from decomposition of tryptophane was therefore present in the inoculum containing the bacteria. Although the bacteria may have been able to synthesize IAA in this medium, their main effect appears to have been spread over a period of time on the plant tissues, when an active substance similar to auxin may have been produced. From the present experiment it would appear that the substance was more active when the bacteria and IAA in lanolin paste were applied at the same time, rather than when the IAA was added four days later.

4.10 Discussion of the Effects of the Bacteria on Older Sunflowers.

These experiments have shown that no callusing could be induced by inoculating intact sunflower plants with E. coli. Since the completion of the experimental work however, there has been a very brief report by Ark and Hunt (1966) that galls formed exceedingly slowly on sunflower plants inoculated with cultures of E. coli, E. intermedia and E. mutabilis, suspended in human plasma. With Datura plants spongy cushions developed on the leaves within a week of inoculation. These later became firm rough nodules while tumours were formed on the inoculated stems of such plants. In

this work, no controls of plants inoculated only with human plasma were prepared, but it was found that ground-up cancer tissue and cancer blood specimens inoculated into Datura induced lesions identical to those produced by the bacteria. These observations are therefore directly contrary to the results obtained from the present experimental work, and it remains to be seen whether the effects reported by Ark and Hunt were the result of the bacteria alone or some other factor of the inoculum not found in the present work.

While the experiments showing increased root production in inoculated sunflower plants suggested that an auxin might be produced by E. coli in conjunction with the plant tissues, Experiment 8 showed that the culture of E. coli was unlike an attenuated strain of A. tumefaciens since application of IAA at the cut surface of the decapitated stem did not induce proliferation at the site of bacterial inoculation. However the work clearly showed that E. coli acted synergistically with IAA when applied simultaneously, so that callusing at the cut surface was stimulated.

The formation of IAA by E. coli from the tryptophane present in nutrient broth was eliminated by the use of the synthetic culture medium which contained only inorganic nitrogen. It was probable that if a stimulating substance similar to an auxin was in fact produced by the bacteria, the plant metabolites would act as the substrate for this formation.

CHAPTER FIVE

GROWTH OF INOCULATED HYPOCOTYL DISKS ON MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF IAA

In the preceding chapter, experiments with older sunflowers were described which showed that inoculation of the hypocotyls with Escherichia coli resulted in an increase in formation of roots from tissues adjacent to the wound. In addition, it was found that the formation of IAA-induced callus at the decapitated stem surfaces was enhanced when the plants were inoculated with bacteria at the same time as the IAA-lanolin paste was applied.

In the experiments using 1 mm-thick sunflower hypocotyl disks described in Chapter 3 it was observed that disks inoculated with E. coli and grown in the light were stimulated to proliferate and produce more roots than uninfected disks. The growth of E. coli-infected disks in the dark was inhibited in comparison with that of uninfected disks.

From the behaviour of both mature sunflower plants and hypocotyl disks in the presence of E. coli it was apparent that some growth factor, similar in some respects to an auxin, was produced by the bacteria either as a result of their own metabolism or in conjunction with the plant tissue. It is known that E. coli can synthesize IAA when grown in a medium containing tryptophane (Hopkins and Cole 1903). However the action of this substance appeared to be more complex than that of an auxin because of its differential effects on hypototyl disks grown in the light and the dark.

EXPERIMENT 9. A COMPARISON OF GROWTH INDUCED BY E. COLI, A. TUMEFACIENS AND IAA

By comparing the growth curves of E. coli-inoculated and uninfected disks grown on media containing varying concentrations of IAA, it was hoped to confirm the similarity of the active substance produced by the bacterium to IAA.

Hypocotyl disks inoculated with Agrobacterium tumefaciens were also prepared, so that their growth responses on media containing different concentrations of IAA could be investigated. Most work on crown gall has been carried out either on whole plants, or on bacteria-free tumour tissue in culture. To the writer's knowledge only two reports have been made on the growth of sunflower hypocotyl segments infected with A. tumefaciens (de Ropp 1948b, 1951c). In the latter report the reactions to infection of segments 5 mm long were recorded with reference to the position of the tissues in the hypocotyl. The only report found which describes growth of infected tissues in which the bacteria were still present, on media containing IAA, was made by Gautheret (1947). In this case the bacteria invaded the medium and the tissues were discarded. It can be assumed that growth of the tissues was inhibited by the bacteria rather than by the presence of auxin in the medium.

Tissues infected with E. coli and A. tumefaciens were grown under similar conditions so that their reactions could be compared. This was considered to be of importance, since so much is known about crown gall tissues, and any similarities between them could be attributed to a similar response.

5.1 Experimental Details.

Disks were grown on de Ropp's medium containing 0.01, 0.1, 1.0

and 10 ppm IAA, and also on the basal medium without added IAA. This range of concentrations was chosen because de Ropp (1947a) found widely differing responses in the growth of sunflower stem segments on media containing a 'low' concentration of 0.01 ppm and a 'high' concentration of 10 ppm IAA respectively.

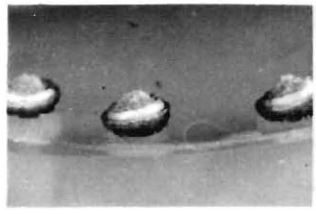
One third of the disks were inoculated with sterile synthetic medium, a third with a 24-hour culture of E. coli in synthetic medium, and the remainder with a 24-hour culture of A. tumefaciens in nutrient broth.

In Experiment 5 (see Chapter 3) only two or four replicates were harvested at any given time for each treatment and difficulty was experienced in the analysis of variance because of these unequal treatment numbers. In the present experiment, ten replicates of every treatment each comprising ten hypocotyl disks were prepared to assess variation within the treatments.

It was also shown in Experiment 5 that the light treatment received by the seedlings affected subsequent growth of the hypocotyl disks. Consequently, in this experiment half of the seedlings were grown in the light and the remainder in the dark before preparation of disks. The disks were also grown in the light or the dark so that all combinations of seedling-light and disk-light treatments were obtained. In Experiment 5 the greatest changes in weights of the disks occurred between three and four weeks. In the present experiment disks from three to six weeks old were harvested to determine whether the sudden increases in weight between three and four weeks continued as the disks aged.

With all the combinations of the various treatments, a

a)



b)

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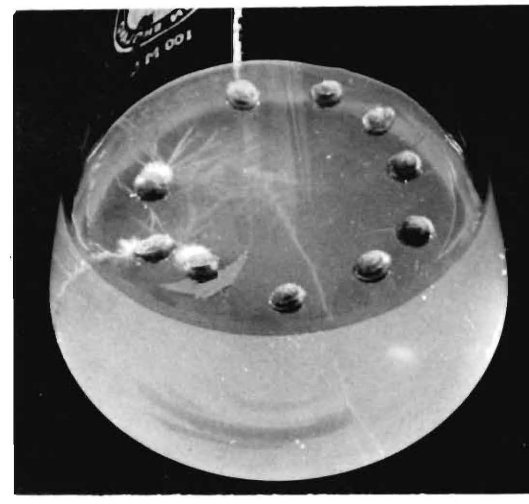


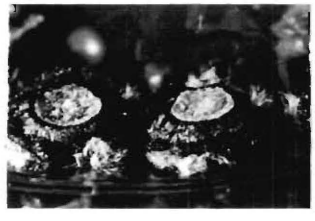
Medium without added IAA (a,c 3 weeks, b 6 weeks)

d)

e)

Medium + 0.01 ppm IAA (d,e 3weeks)

f)



g)

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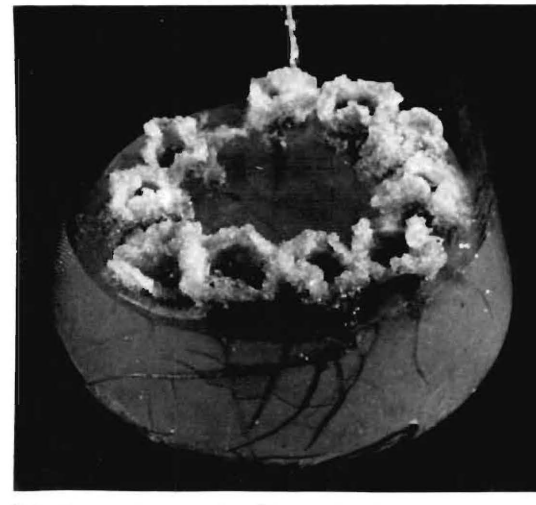


Medium + 0.1ppm IAA (f 6weeks, g,h 3 weeks)

FIGURE 5.1 UNINFECTED DISKS GROWN IN THE LIGHT ON MEDIA CONTAINING 0, 0.01 AND 0.1PPM IAA (Mag. of flasks x1)

factorial experiment of the order 3 x 2 x 2 x 5 was prepared, comprising three different bacterial treatments, two seedling-light treatments, two disk-light treatments, and five media containing different concentrations of IAA, with ten replicates of each treatment. These were harvested weekly for four weeks. The experiment was carried out in four parts, each combination of seedling-light and disk-light treatments being prepared separately. In all, 2,400 flasks, each containing ten hypocotyl disks were prepared. Both fresh and dry weights of the disks and associated root tissues in each flask were recorded, and photographs were taken of many of the treatments. The type of proliferation and number of roots formed from each disk were recorded at harvesting.

Results.

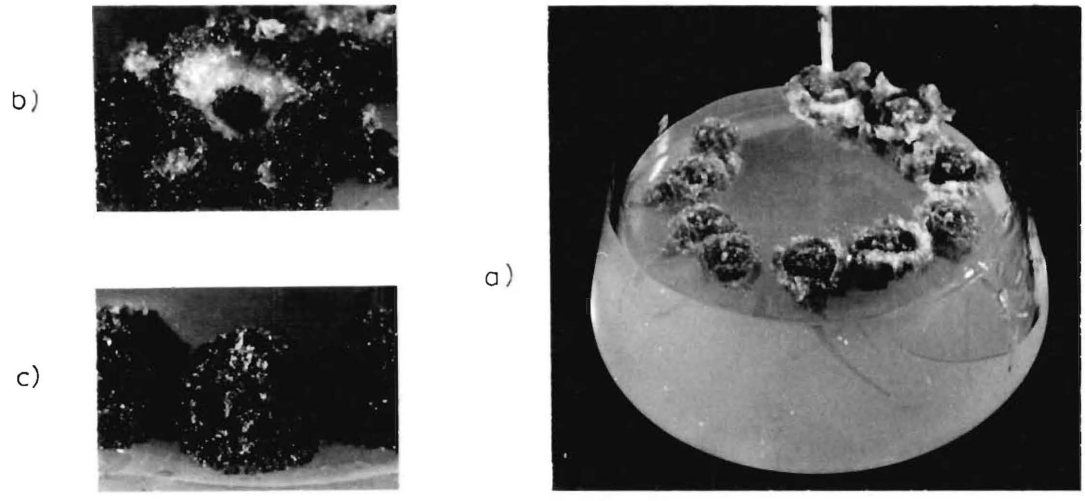
5.2 Morphological Observations of the Hypocotyl Disks.

Because 400 disks of varying ages were described in each treatment, only general trends in the gross morphology of the disks have been noted below. The descriptions of all the treatments have been summarized in tabular form in Table 1, Appendix III.

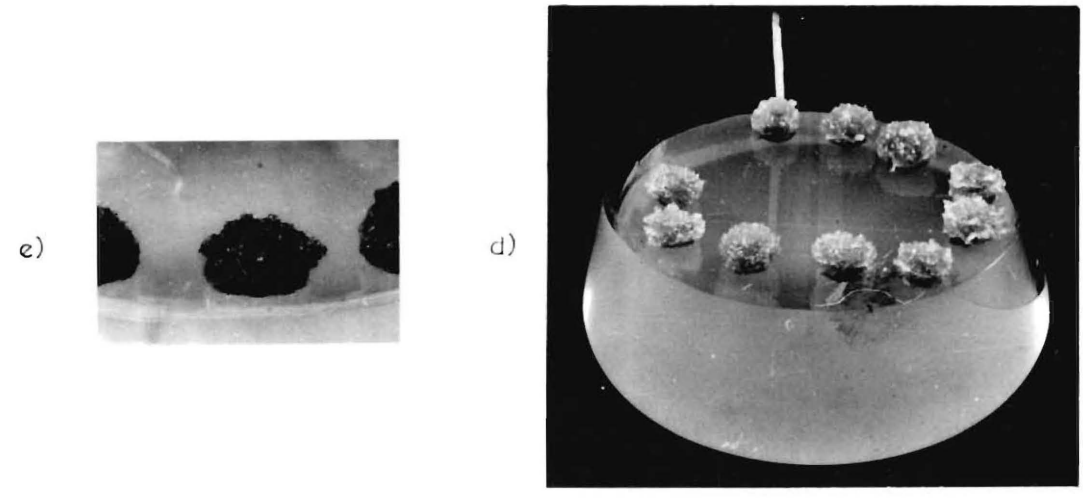
Growth in the Light

5.3 Uninfected Disks.

a) Medium without added IAA. Most disks remained green throughout the six weeks of growth, and proliferated slightly from both upper and lower surfaces (Figure 5.1a). Occasional disks expanded greatly from the upper surface through water uptake, and in these the tissues soon became brown (Figure 5.1b). In each flask about half the disks prepared from seedlings grown in the light formed



Medium + 1.0 ppm IAA (a 3 weeks, b, c 6 weeks)



Medium + 10 ppm IAA (d 3 weeks, e 6 weeks)

FIGURE 5.2 UNINFECTED DISKS GROWN IN THE LIGHT ON MEDIA CONTAINING 1.0 AND 10 PPM IAA (Mag. of flasks x1)

one or two roots (Figure 5.1c). All disks from seedlings grown in the dark formed roots.

b) Medium + 0.01 ppm IAA. The disks increased in size and proliferated more from the lower surface than when no IAA was present (Figure 5.1d). Loose parenchymatous tissue was formed which pushed out the epidermis at right angles to its normal position. The vascular tissue at the upper surface of the disks also proliferated. Again a difference existed between the rooting response of disks prepared from seedlings grown in different light conditions. Where seedlings had been grown in the light, most disks produced two or three roots, but disks from dark-grown seedlings formed up to six roots each. Irrespective of the light treatment of the seedlings, these disks formed more roots than those grown on the medium without IAA (Figure 5.1e).

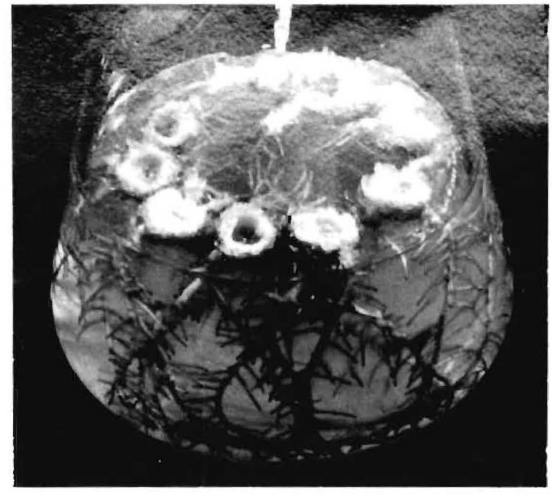
c) Medium + 0.1 ppm IAA. With a tenfold increase in the concentration of IAA in the medium to 0.1 ppm, most disks continued to proliferate from the lower surface (Figure 5.1f). There was, however, a tendency for the tissues in a few of the disks to become hyperhydric (Figure 5.1g), with more cell expansion than cell division occurring. Such disks frequently became brown and necrotic. Numbers of short straight roots with few laterals were formed from the hyperhydric tissues (Figure 5.1h). These contrasted with the long branching roots produced when lower concentrations of IAA were present in the medium. There were no apparent differences between disks from seedlings which had been grown in different light conditions.

d) Medium + 1.0 ppm IAA. Disks were more hyperhydric than those

a)

b)

c)



Medium without added IAA (a,c 3 weeks, b 6 weeks)

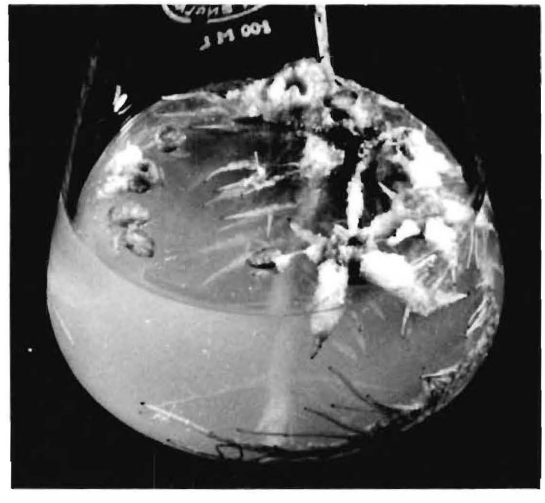
d)

e)

Medium + 0.01 ppm IAA (d,e 3 weeks)

f)

g)



Medium + 0.1 ppm IAA (f,g 3 weeks)

FIGURE 5.3 DISKS INOCULATED WITH E.COLI AND GROWN IN THE LIGHT ON MEDIA CONTAINING 0, 0.01 AND 0.1 PPM IAA (Mag. of flasks x 1)

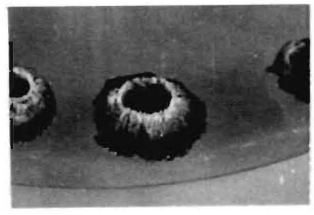
grown on the lower concentrations of IAA (Figure 5.2a). The tissues expanded from either the lower surface (Figure 5.2b), or from the upper surface (Figure 5.2c). Short roots with few if any laterals were formed from the disks which did not show excessive water uptake.

e) Medium + 10 ppm IAA. The growth of disks was inhibited in comparison with that at lower concentrations of IAA (Figure 5.2d). Disks expanded more at the upper than the lower surface, and the tissues quickly became brown and necrotic (Figure 5.2e). Only occasional short roots were formed.

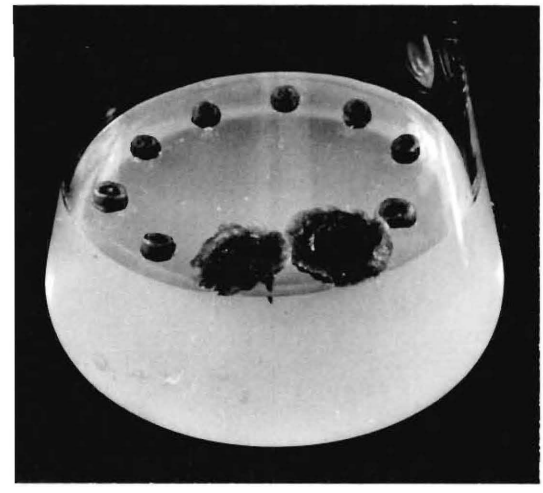
5.4 Disks Inoculated with E. coli.

a) Medium without added IAA. As described in Experiments 4 and 5 (Chapter 3) E. coli-inoculated disks grown on de Ropp's medium reacted to the presence of the bacteria by the formation of a mass of parenchymatous tissue from the lower surface, which pushed out the epidermis at right angles to its usual position (Figure 5.3a). The epidermis was usually not visible after six weeks' growth, because of the amount of proliferation (Figure 5.3b). When seedlings from which the disks were prepared had been grown in the light, the majority of disks formed one or two long roots. Disks from dark-grown seedlings produced an increasing number of roots with age, five or six roots eventually being formed (Figure 5.3c). In many cases the roots were covered with a loose parenchymatous tissue, similar to that formed from the proliferating tissues at the base of disks. The inhibiting effects of light treatment of the seedlings on the subsequent formation of roots in uninfected disks was overcome to a certain extent by

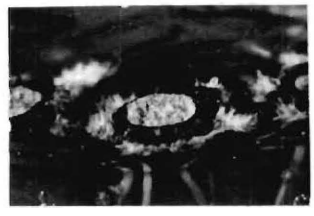
a)



b)

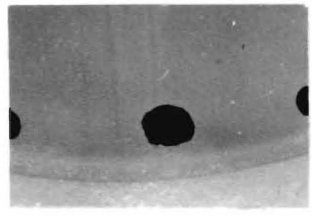


c)

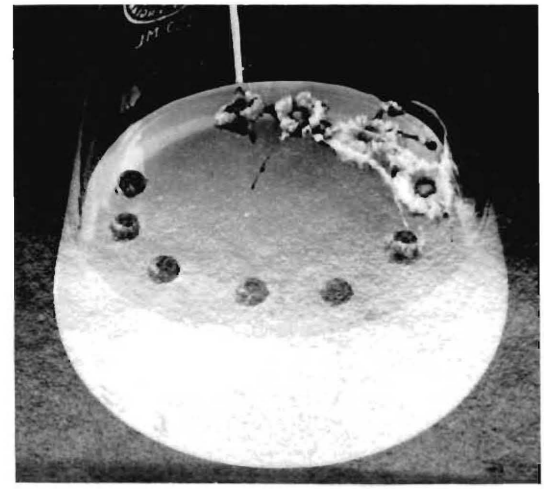


Medium + 1.0 ppm IAA (a, b 3 weeks, c 6 weeks)

d)



e)



f)

Medium + 10 ppm IAA (d, e, f 3 weeks)

FIGURE 5.4 DISKS INOCULATED WITH E.COLI AND GROWN IN THE LIGHT ON MEDIA CONTAINING 1.0 AND 10 PPM IAA (Mag. of flasks x1)

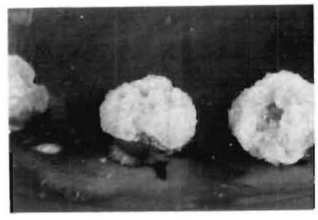
inoculation with E. coli.

b) Medium + 0.01 ppm IAA. The lower tissues of disks inoculated with E. coli were stimulated to proliferate more than in the absence of IAA (Figure 5.3d). Each disk formed at least two roots, and up to five were often present. Loose parenchyma formed under the influence of the bacteria again covered the main roots (Figure 5.3e). No differences could be detected between disks from seedlings which had received the differing light treatments. Compared with uninfected disks grown on the same medium, those inoculated with E. coli proliferated much more from the lower surface. There was no evidence that the vascular tissues had proliferated from the upper surface in the same way as in uninfected disks.

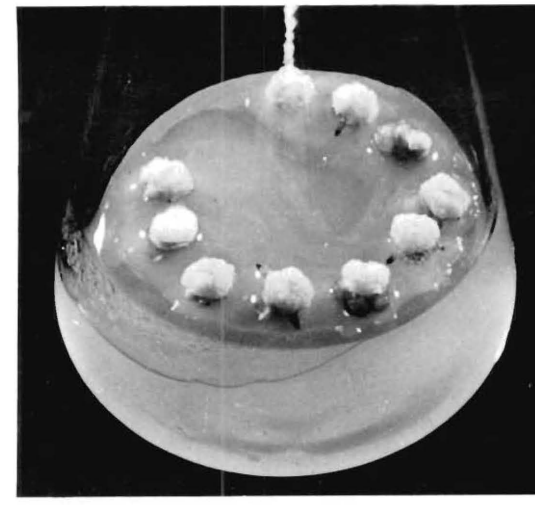
c) Medium + 0.1 ppm IAA. Most disks proliferated from the lower surface and were of a similar size to those grown with the lower IAA concentration (Figure 5.3f). The tissues however, tended to be a little more hyperhydric. Up to five or six roots were formed from each disk, and these were sometimes coated with loose parenchyma. In many disks from dark-grown seedlings, little growth occurred and the tissues quickly became brown and necrotic (Figure 5.3g). These disks sometimes formed roots which became coated with loose parenchyma. Disks from light-grown seedlings were all of a similar size to uninfected disks grown on the same medium.

d) Medium + 1.0 ppm IAA. On this medium disks inoculated with E. coli showed a higher death rate than those grown on lower concentrations. Disks which became necrotic usually swelled a little before death (Figure 5.4a) although they occasionally proliferated from the lower surface (Figure 5.4b). Some healthy disks prolife-

a)

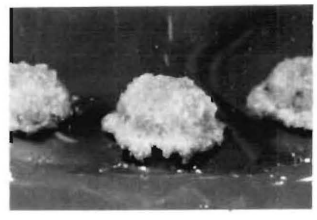


b)

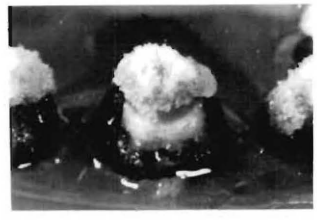


Medium without added IAA (a, b 3 weeks)

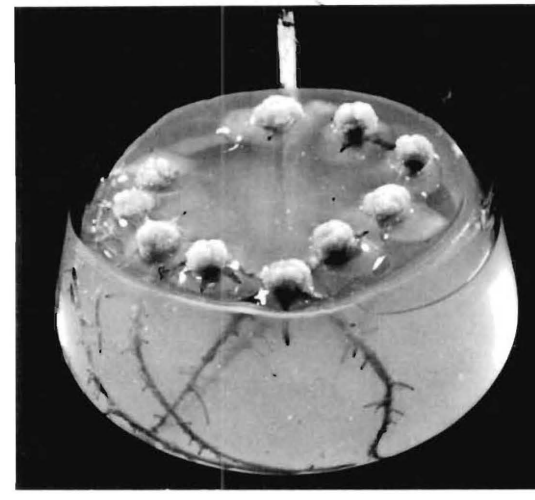
c)



d)

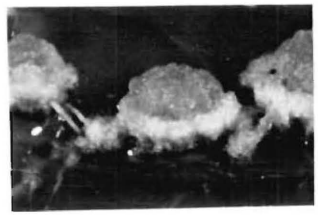


e)

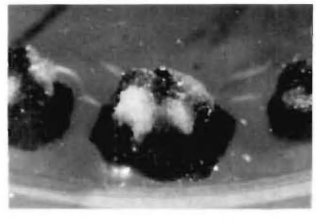


Medium + 0.01 ppm IAA (c, e 3 weeks, d 6 weeks)

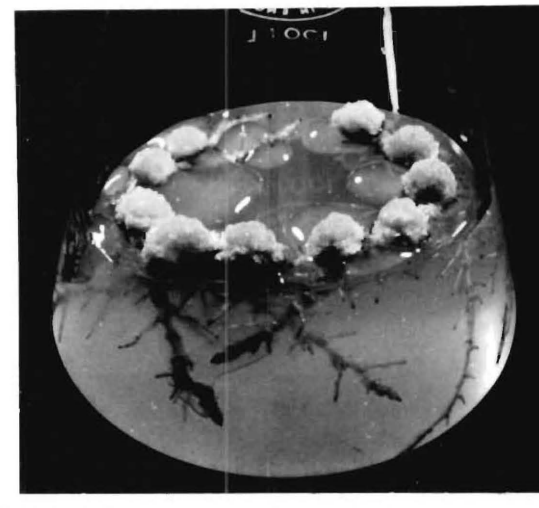
f)



g)



h)



Medium + 0.1 ppm IAA (f, h 3 weeks, g 6 weeks)

FIGURE 5.5 DISKS INOCULATED WITH A.TUMEFACIENS AND GROWN IN THE LIGHT ON MEDIA CONTAINING 0, 0.01 AND 0.1 PPM IAA (Mag. of flasks x1)

rated but they did not form as much tissue as those grown on lower concentrations of IAA (Figure 5.4c), and they formed five or six short roots. The general reaction of early death in disks inoculated with E. coli and grown on medium containing 1 ppm IAA, contrasted with that of uninfected disks grown under the same conditions. The latter expanded and became hyperhydric.

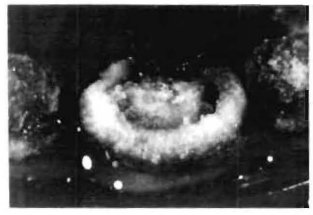
e) Medium + 10 ppm IAA. Even greater inhibition of tissue expansion occurred on this medium. Disks usually died after tissues had expanded a little (Figure 5.4d). In the few cases when roots were formed, they were very short (Figure 5.4e). Occasionally disks remained alive and proliferated from the lower surface, and a tangled mass of short roots sometimes formed (Figure 5.4f).

5.5 Disks Inoculated with A. tumefaciens.

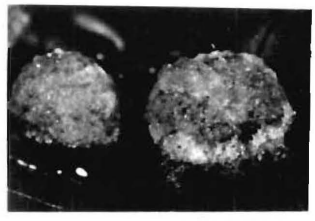
a) Medium without added IAA. In contrast to uninfected or E. coli-inoculated disks, those inoculated with A. tumefaciens proliferated from the upper surface, where most of the tissues within the epidermis contributed to form a compact white mass of tissue (Figure 5.5a). These disks were larger than uninfected disks, but they did not reach the diameter of E. coli-inoculated disks. In most cases the disks produced up to five or six very short roots with blackened tips (Figure 5.5b). It appeared that root initiation had been stimulated but that further growth was in some way inhibited through the action of the bacteria. The number of roots formed was not affected by the seedling-light treatment.

b) Medium + 0.01 ppm IAA. When disks inoculated with A. tumefaciens were grown on this medium, most proliferation again occurred from the upper surface. The tissues immediately inside the epidermis

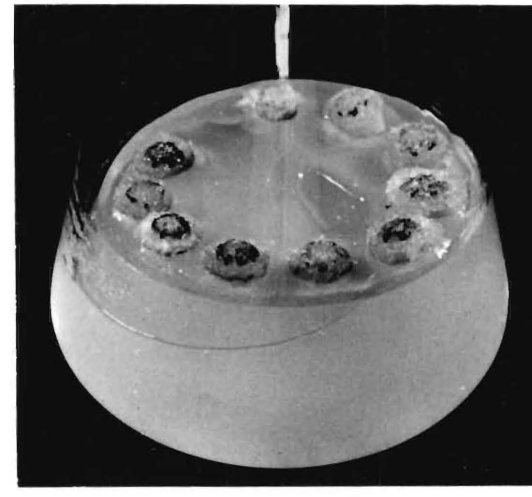
a)



b)

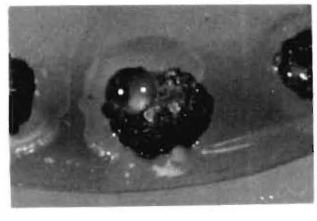


c)

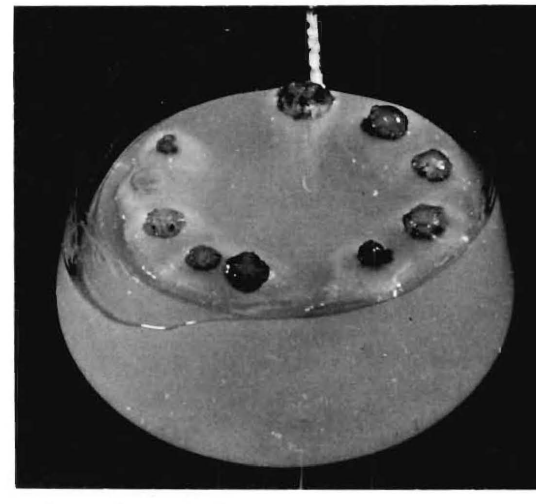


Medium + 1.0 ppm IAA (a, b, c 3 weeks)

d)



e)



Medium + 10 ppm IAA (d, e 3 weeks)

FIGURE 5.6 DISKS INOCULATED WITH A.TUMEFACIENS AND GROWN IN THE LIGHT ON MEDIA CONTAINING 1.0 AND 10 PPM IAA (Mag. of flasks x 1)

were however stimulated in comparison with disks grown on medium to which no IAA had been added (Figure 5.5c). In some cases, tissues at the lower surface were also stimulated to divide (Figure 5.5d). These disks were no larger than those grown without added IAA. However, longer roots were formed from more of the disks than when no IAA was present in the medium, particularly when dark-grown seedlings were used. The root tips were again blackened (Figure 5.5e).

c) Medium + 0.1 ppm IAA. On this medium disks were much larger than those grown on the medium containing 0.01 ppm IAA and showed increased proliferation of the cortical tissues near the upper surface, although there was no noticeable cell division from the lower surface (Figure 5.5f). In many cases a necrotic area developed around the rim of the disks where the bacteria had formed a viscous coating. Occasionally disks became almost entirely necrotic (Figure 5.5g). Most formed up to five or six roots, again with blackened tips (Figure 5.5h).

d) Medium + 1.0 ppm IAA. The majority of disks became hyperhydric, although some tissue around the edges proliferated (Figure 5.6a). These disks retained features characteristic of infection with A. tumefaciens in spite of their hyperhydricity, and were usually smaller than uninfected disks grown under the same conditions. Some disks did not absorb as much water, and proliferated from the upper surface forming compact firm tissue (Figure 5.6b). These disks produced numbers of roots. Again, disks tended to become necrotic, particularly at their outer edges, where a viscous coating of bacteria had formed (Figure 5.6c).

e) Medium + 10 ppm IAA. When disks inoculated with A. tumefaciens were grown on this medium, greater inhibition of growth occurred than in uninfected disks, and this was followed by necrosis. The disks proliferated a little from the upper surface before dying, and were larger than those inoculated with E. coli and grown under the same conditions (Figure 5.6d). None of the disks formed roots. With this medium in particular, the bacteria were stimulated to divide, so that most of the surface of the medium was covered by a viscous fluid (Figure 5.6e).

5.6 Comparisons between the Bacterial Treatments, Grown with Differing Concentrations of IAA in the Light.

Although enlargement of existing structures occurred, there was little absolute change in the gross morphology of hypocotyl disks three to six weeks after inoculation. In some treatments, for example uninfected disks grown on medium containing 0.01 ppm IAA, more roots were formed with aging, while in others necrosis occurred. Generally the trends in growth of the disks occurring during thefirst three weeks were followed for the remaining time before harvesting.

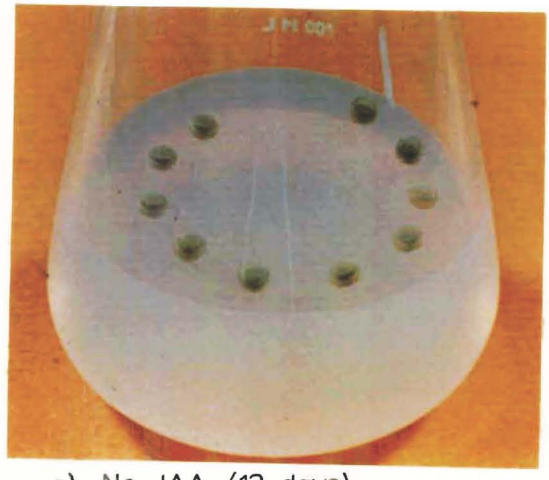
The effect of seedling-light treatment on the subsequent development of the disks, was most evident in uninfected disks grown on media without IAA, or containing 0.01 ppm. When the seedlings had been grown in the dark, disks showed a greater tendency towards formation of roots than those from light-grown seedlings. This inhibition of rooting in disks from light - grown seedlings, was overcome to a great extent by inoculation

with E. coli.

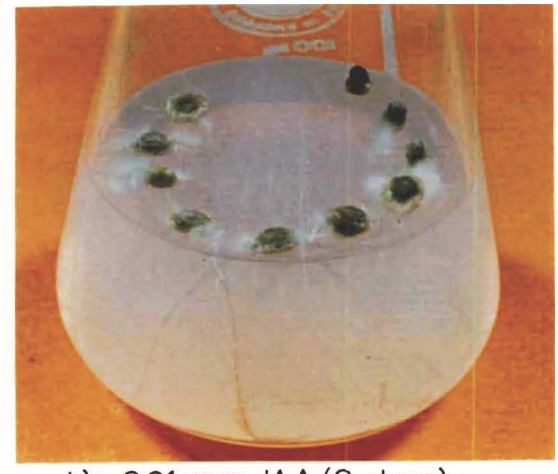
With increasing concentration of IAA, uninfected disks first formed more roots and proliferated more from the lower surface, but at higher concentrations, the disks expanded and became hyperhydric, forming only short roots. At the highest concentration, the disks were hyperhydric but were not as well expanded, and root formation was inhibited.

Disks inoculated with E. coli also showed a range of variation with increasing IAA concentration. With the lowest concentrations of IAA they proliferated enormously and a number of roots were formed. Increasing concentrations, however, caused death after the tissues had expanded only a little. There was little tendency for disks inoculated with E. coli to become hyperhydric with the higher concentrations of IAA, as did uninfected disks.

At all concentrations of IAA, disks infected with A. tumefaciens proliferated from the upper surface. At low concentrations longer roots were formed than when no IAA was present in the medium. At higher concentrations, some disks became hyperhydric before dying. It is interesting to note that although crown gall tissues are hyperauxinic (Kulescha and Gautheret 1948), root growth was increased by the addition of IAA. Gautheret (1959) has shown that for normal tissues in culture, low concentrations of auxin stimulate root production, but with increasing concentrations, tissues become hyperhydric. It would be expected that the addition of IAA to crown gall tissues would inhibit, rather than stimulate root growth, but this was not the case with disks grown on medium containing 0.01 ppm IAA. The root tips became



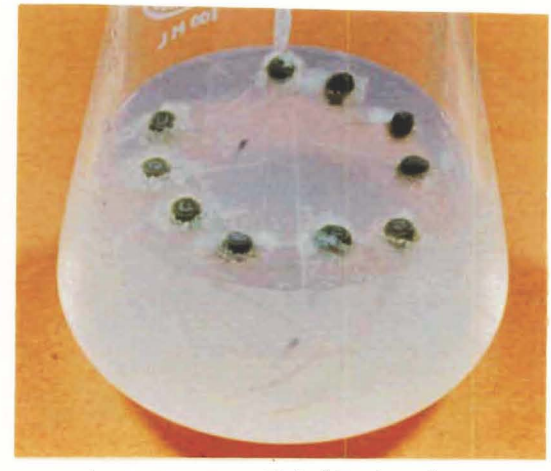
a) No IAA (13 days)



b) 0.01 ppm IAA (8 days)

Uninfected disks

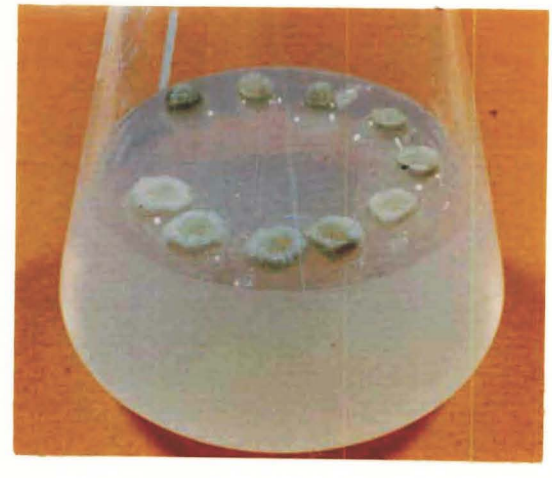
c) No IAA (12 days)



d) 0.01 ppm IAA (7 days)

Disks inoculated with E.coli

e) No IAA (12 days)



f) 0.01 ppm IAA (7 days)

Disks inoculated with A.tumefaciens

FIGURE 5.7 INOCULATED DISKS GROWN IN THE LIGHT ON MEDIA CONTAINING 0 AND 0.01 PPM IAA (Mag. x 1)

blackened, whether or not there was any IAA in the medium. It was apparent that the bacteria inhibited the further development of the initiated roots, although this was overcome to a limited extent with the addition of IAA. One can only conclude that the inhibitory action on root growth is in no way connected with the auxin content of the infected tissues, and that when low concentrations of IAA are added to such tissues, this overcomes the inhibition of root growth .

Disks inoculated with E. coli were similar in form to uninfected disks, but they proliferated more when grown on media without IAA, with 0.01 ppm IAA or with 0.1 ppm IAA (Figure 5.7 a,b,c,d). With further increases in concentration, the treatments diverged; uninfected tissues became hyperhydric, while increasing numbers of E. coli-inoculated disks died. The response of disks to inoculation with A. tumefaciens was quite different from that of E. coli-infected disks (Figure 5.7e,f). Both bacterial treatments thus have a characteristic effect on sunflower hypocotyl disks grown in the light.

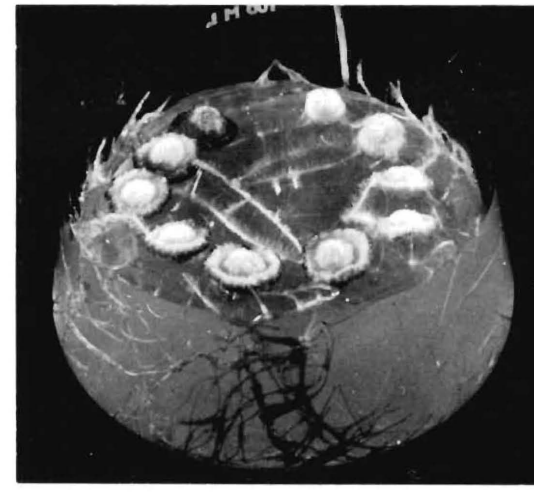
Growth in the Dark

5.7 Uninfected Disks

a) Medium without added IAA. Uninfected disks grown on de Ropp's medium without added IAA were similar in form to disks grown in the light on the same medium (Figures 5.8a, b). As with disks grown in the light, the rooting response appeared to be dependent on the seedling-light treatment. Disks from dark-grown

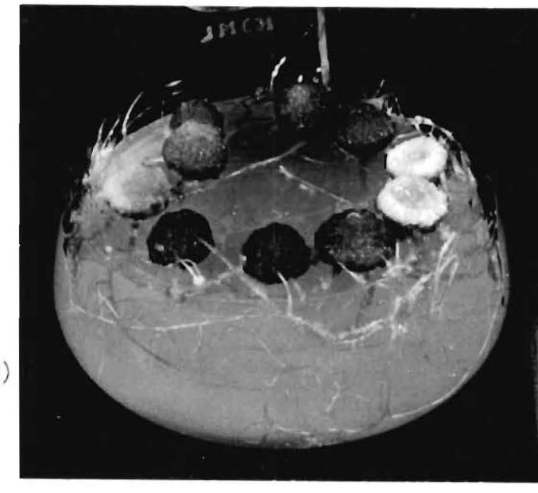
a)

b)



Medium without added IAA (a, b 4 weeks)

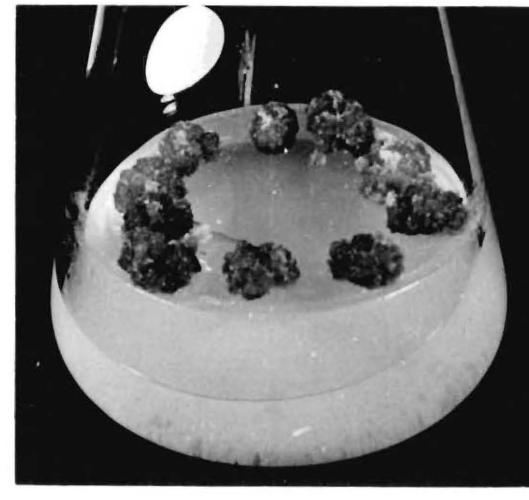
c)



d)

Medium + 0.01 ppm IAA (c, d 4 weeks)

e) Medium + 0.1 ppm IAA (4 weeks)



f) Medium + 1.0 ppm IAA (4 weeks)

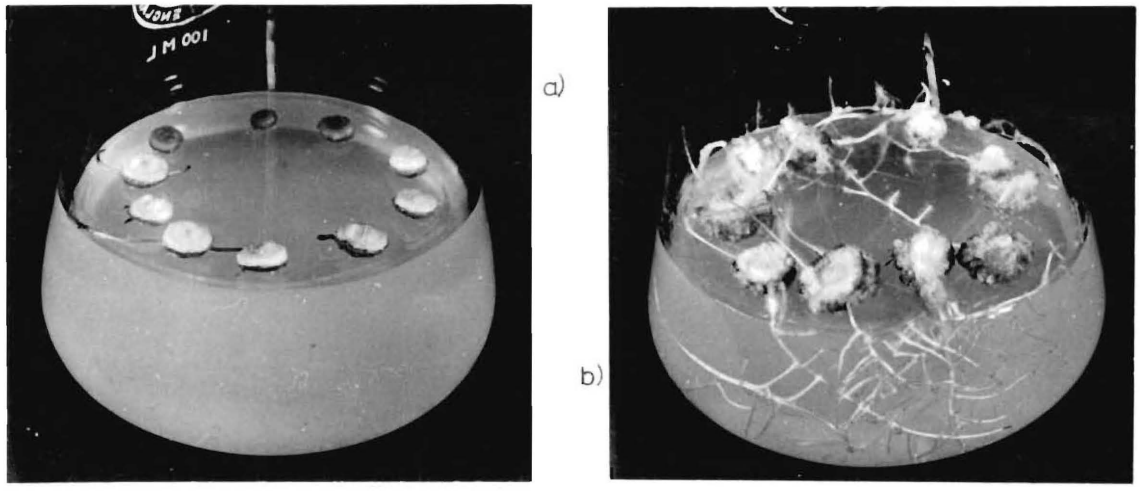
FIGURE 5.8 UNINFECTED DISKS GROWN IN THE DARK ON MEDIA CONTAINING 0, 0.01, 0.1 AND 1.0 PPM IAA (Mag of flasks x 1)

seedlings usually formed four to six roots each, but only two or three roots were produced in those from light-grown seedlings; in both cases the long fine roots formed many laterals. Occasionally the tissues of the upper surface of disks expanded by water uptake more than by cell division, and became brown. Usually no roots were formed from such disks.

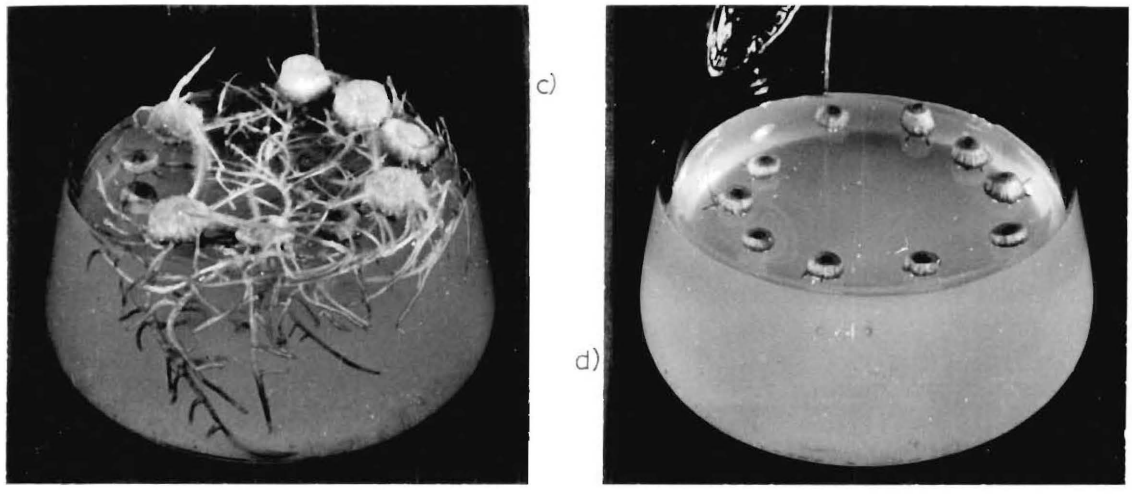
b) Medium + 0.01 ppm IAA. More proliferation occurred from both upper and lower surfaces of the disks than when no IAA was present. The latter tissues often pushed out the epidermis at right angles to its usual position (Figure 5.8c). More roots were formed than when no IAA was included in the medium. There was no difference between disks prepared from seedlings receiving different light treatments. With age, disks tended to become brown at the lower surface. Occasionally disks became hyperhydric and the tissues at the upper surface expanded as described for disks grown without IAA (Figure 5.8d).

c) Medium + 0.1 ppm IAA. When grown on this medium uninfected disks again proliferated from both surfaces. These tissues were more hyperhydric than disks grown with lower concentrations of IAA, and frequently became greenish-brown with cessation of growth (Figure 5.8e). The hyperhydric disks formed short roots lacking a well defined structure. Where the tissues remained firm, five or six long roots were formed from each disk.

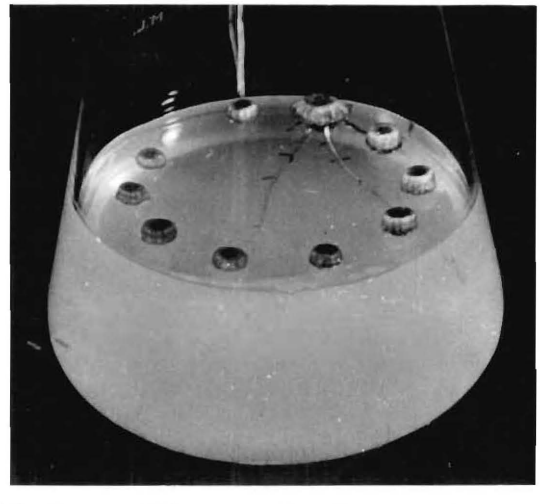
d) Medium + 1.0 ppm IAA. The disks expanded mostly from the upper surface. The tissues were hyperhydric, and it is doubtful whether much growth occurred after the initial expansion and absorption of water (Figure 5.8f). In the few cases where roots



Medium without added IAA (4 weeks)



Medium + 0.01 ppm IAA (4 weeks)



e) Medium + 0.1 ppm IAA (4 weeks)

FIGURE 5.9 DISKS INOCULATED WITH E.COLI AND GROWN IN THE DARK ON MEDIA CONTAINING 0, 0.01 AND 0.1 PPM IAA (Mag. x 1)

were formed they lacked definite structure.

e) Medium + 10 ppm IAA. At this concentration of IAA, the tissues expanded and absorbed water, but disks were smaller than those grown on media containing lower concentrations of IAA. Few if any roots were formed.

5.8 Disks Inoculated with E. coli.

a) Medium without added IAA. In the dark, the growth of disks inoculated with E. coli was quite different from that of similarly inoculated disks grown in the light. When grown on de Ropp's medium, without added IAA, E. coli-inoculated disks usually expanded a little but there was no proliferation. With age, disks frequently became brown and necrotic (Figure 5.9a). One or two short roots and occasionally up to five, were formed from some of the disks. One or two flasks in every ten contained disks which proliferated quite markedly from the lower surface, in contrast with the majority in which no proliferation occurred. The tissues formed were larger than those of uninfected disks and numbers of long much-branched roots were produced (Figure 5.9b).

b) Medium + 0.01 ppm IAA. The disks showed a much higher tendency to proliferate and form roots than those grown without IAA. When disks were prepared from dark-grown seedlings, more proliferated in each flask than when they were prepared from seedlings grown in the light (Figure 5.9c). Four to six roots, forming a tangled mass, were usually produced from each disk, but these lacked the coating of parenchyma found in similarly-treated disks grown in the light. Those not proliferating in this manner expanded only a little before the tissues became necrotic, and a few short roots were formed (Figure 5.9d).

c) Medium + 0.1 ppm IAA. Disks grew only a little before becoming brown and necrotic, but some disks formed up to four or five short roots before death (Figure 5.9e).

d) Medium + 1.0 ppm IAA. The same reaction occurred in disks grown on this medium, although fewer roots were formed.

e) Medium + 10 ppm IAA. At concentrations of 10 ppm IAA, disks grew only a little before becoming necrotic; no roots were formed.

5.9 Disks Inoculated with A. tumefaciens.

a) Medium without added IAA. When no IAA was included in the medium, disks inoculated with A. tumefaciens proliferated mainly from the upper surface, although some proliferation from the lower surface occurred. Many of the disks produced three to five roots which grew little and showed blackening of the root tips. The form of growth of these disks was similar to those grown in the light, although they were not as large.

b) Medium + 0.01 ppm IAA. Growth of disks was similar to that when no IAA was present, although usually four to six longer roots were formed.

c) Medium + 0.1 ppm IAA. Most A. tumefaciens-inoculated disks grown on this medium became necrotic after having proliferated a little from the upper surface. Some disks formed up to six roots.

d) Medium + 1.0 ppm IAA. With this concentration of IAA, none of the disks remained alive although most had proliferated a little before becoming necrotic; a few formed short roots.

e) Medium + 10 ppm IAA. All the disks died after expanding

only a little. As in the light, A. tumefaciens multiplied on de Ropp's medium, such that with higher concentrations of IAA the disks were covered completely in many cases.

5.10 Comparisons between the Bacterial Treatments, Grown with Differing Concentrations of IAA in the Dark.

As with disks grown in the light, little change occurred in the pattern of growth of the disks between three and six weeks. The seedling-light treatment influenced the subsequent growth of uninfected disks on medium without added IAA as described for light-grown disks. An unexpected effect of the seedling-light treatment was noted. On the medium containing 0.01 ppm IAA the proportion of disks from dark-grown seedlings proliferating after inoculation with E. coli was greater than that of disks from seedlings grown in the light.

Uninfected disks proliferated more and produced a greater number of roots when low concentrations of IAA were present in the medium. With increasimg concentrations of IAA water uptake increased and there was a reduction in root formation until with 10 ppm IAA, there was little growth and no roots formed.

Some E. coli-infected disks grown on media containing 0.01 ppm IAA, or without added IAA, proliferated more than uninfected disks grown under the same conditions. However, the usual action of the bacteria was to inhibit growth of the disks as compared with that of the uninfected ones. With higher concentrations of IAA, this inhibition increased although roots were still initiated at concentrations of 1.0 ppm IAA. Little

expansion of infected disks occurred on medium containing 10 ppm IAA. At no time did E. coli-infected disks, grown in the dark, become hyperhydric when IAA was present in the medium. It was apparent that some inhibiting growth factor from the bacteria was overcome by concentrations of 0.01 ppm IAA, but was reinforced by higher concentrations of auxin so that generally growth was retarded in comparison with similarly grown uninfected disks.

Low concentrations of IAA caused a slight increase in the amount of proliferation and root formation of A. tumefaciens-inoculated disks. At higher concentrations growth was inhibited and, as in disks infected with E. coli, necrosis soon occurred. Throughout the range of concentrations of IAA, growth of A. tumefaciens-inoculated disks was less than that of uninfected disks grown under comparable conditions, and fewer roots were formed.

5.11 Comparisons Between Similarly Treated Light and Dark Grown Disks.

When disks were grown in the dark, death occurred at lower concentrations of IAA than when they were grown in the light. This was true of both infected and uninfected disks. Uninfected disks became hyperhydric at lower concentrations of IAA in the dark, and did not become as large as comparable disks grown in the light. When no IAA was added to the medium more roots were formed from disks grown in the dark than from light-grown disks. In both the light and the dark, seedling-

light treatment was responsible for a difference in the number of roots formed by uninfected disks.

Disks inoculated with A. tumefaciens showed very similar reactions in the light and dark, to increasing amounts of IAA in the medium. As with uninfected disks, however, less growth occurred in the dark and disks died at lower concentrations of IAA than those grown in the light. Disks grown in the light from dark-grown seedlings, were stimulated to form longer roots when grown on medium containing 0.01 ppm IAA. As has been noted, this stimulation by low concentrations of IAA is contrary to that expected with hyperauxinic tissues.

With E.coli-inoculated disks, growth reactions were completely different in different light conditions. In the light, disks were stimulated to proliferate at concentrations of IAA up to 0.1 ppm; disks died after only a little growth with higher concentrations of auxin. In the dark, growth of infected disks was mainly inhibited throughout the range of IAA concentrations. However, many of the disks prepared from dark-grown seedlings proliferated when grown on medium containing 0.01 ppm IAA. E. coli-infected disks in the dark grew even less before death at the higher concentrations of IAA than did disks grown in the light.

Thus characteristic reactions occurred when disks inoculated with either E. coli or A. tumefaciens were grown in the light and the dark, on media containing different concentrations of IAA. These reactions differed from those of uninfected disks.

TABLE 5.1 EFFECT OF IAA AND SEEDLING-LIGHT TREATMENT ON THE GROWTH* OF INOCULATED DISKS IN THE LIGHT AND THE DARK

SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT															
		Light						Dark						Sum of disk-light treatments			
		IAA CONCENTRATION (ppm)					Sum of IAA Treatments (TOTAL)	IAA CONCENTRATION (ppm)					Sum of IAA Treatments (TOTAL)	IAA CONCENTRATION (ppm)					Sum of IAA Treatments (TOTAL)
		0	0.01	0.1	1.0	10		0	0.01	0.1	1.0	10		0	0.01	0.1	1.0	10	
Light	Synthetic medium	192	355	401	286	110	1344	365	345	342	245	84	1381	557	700	743	532	194	2725
	E. coli	342	398	352	74	26	1192	87	69	32	16	11	215	429	467	384	90	37	1407
	A. tumefaciens	97	127	140	144	55	563	132	136	166	88	25	547	229	263	306	232	80	1110
	Sum of bacterial treatments	631	880	893	504	191	3099	584	550	540	349	120	2143	1215	1430	1433	853	311	5242
Dark	Synthetic medium	425	499	543	429	311	2207	236	279	230	216	64	1025	661	778	773	645	375	3232
	E. coli	504	495	270	90	32	1391	27	135	25	17	11	215	531	630	295	107	43	1606
	A. tumefaciens	158	392	196	148	97	991	114	121	105	99	15	454	272	513	301	247	112	1445
	Sum of bacterial treatments	1087	1386	1009	667	440	4589	377	535	360	332	90	1694	1464	1921	1369	999	530	6283
Sum of Seedling-light Treatments	Synthetic medium	617	854	944	715	421	3551	601	624	572	461	148	2406	1218	1478	1516	1176	569	5957
	E. coli	846	893	622	164	58	2583	114	204	57	33	22	430	960	1097	679	197	80	3013
	A. tumefaciens	255	519	336	292	152	1554	246	257	271	187	40	1001	501	776	607	479	192	2555
	Sum of bacterial treatments	1718	2266	1902	1171	631	7688	861	1085	900	481	210	3837	2679	3351	2802	1852	841	11525

* Expressed as the total over four weeks of the average final fresh weight/average initial weight x10

5.12 Analysis of the Fresh and Dry Weights of the Disks.

An analysis of variance of the initial fresh weights of hypocotyl disks, measured at their time of preparation from seedlings grown in different light conditions, showed that a highly significant difference at the 1% level existed between the disks prepared at different times during the course of the experiment (Table 2, Appendix III). This difference was probably the result of carrying experimentation over a period of one year. All seeds were selected for uniformity from a single batch. Those for the second half of the experimental work were less uniform than those for the first half, because fewer seeds were available for selection as the experiment progressed.

Because of the variability in the initial weights of the disks, the average final weights for each treatment were divided by the average initial weights of the disks. Analysis of variance using these adjusted weights was made on the University of Canterbury IBM 1620 computer. Fresh and dry weights were analysed separately, but because of their similarity, only the changes in fresh weight of the disks three to six weeks old will be discussed. The results are summarized in Table 5.1 and in Tables 3, 4, 5, 6, 7, Appendix III.

Very highly significant differences were found to exist among the main treatments. Disks three to six weeks old prepared from dark-grown seedlings were generally heavier than those from seedlings grown in the light (6283 and 5242 respectively: see Table 5.1). Thus the seedling-light treatment had a

significant effect on the subsequent growth of the disks. When the ratios of the final to initial weights of light and dark-grown disks were summed those grown in the light were over twice as heavy as dark-grown disks (7688 and 3837 respectively). The light treatment of the disks after inoculation had a more highly significant effect on growth than the seedling-light treatment prior to inoculation of the disks. Uninfected disks were almost double the weight of disks infected with E. coli, while A. tumefaciens-infected disks were lighter than both these (5957, 3013 and 2555 respectively). Each bacterial treatment therefore influenced growth in a different way. Finally, when growth at different concentrations of IAA was compared, 0.01 ppm and 0.1 ppm IAA stimulated growth above the level of the control treatment, while at higher concentrations growth was comparatively inhibited. From Table 5.1 it can be seen that the adjusted weights with increasing IAA concentration were 2679, 3351, 2802, 1852 and 841. The IAA content of the medium also significantly influenced the weights of the hypocotyl disks.

From this preliminary analysis it was obvious however that no single factor had a specific effect on all the disks, and the interactions between the treatments were therefore studied. Only statistically significant interactions have been described, although the levels of significance varied throughout the four weeks of harvesting (Table 7, Appendix III).

The main interaction was between bacterial and disk-light treatments. When totalled over the various concentrations of

IAA, uninfected disks grown in the light were about one and a half times as heavy as those inoculated with E. coli which in turn were about twice as heavy as A. tumefaciens-infected disks (3551, 2583, and 1554 respectively: see Table 5.1). In the dark, uninfected disks were about five times as heavy as those inoculated with E. coli (2406 and 430 respectively in Table 5.1). In this case, disks infected with A. tumefaciens were heavier than E. coli-inoculated disks. However, the presence of varying concentrations of IAA in the medium was important in determining the way in which different bacterial treatments reacted when grown under the different light conditions.

5.13 Interactions Between Bacterial, Disk-light and IAA Treatments.

In the light, (Figure 5.10a and b) growth of uninfected disks was stimulated with concentrations of 0.01 and 0.1 ppm IAA, but with higher concentrations, the weights decreased to less than those of disks grown without IAA. By comparison, disks inoculated with E. coli were heavier than uninfected disks when no IAA was present in the medium, and were stimulated by the addition of 0.01 ppm IAA, so that they remained heavier than uninfected disks. With higher concentrations of IAA, the growth of E. coli-infected disks was inhibited in comparison with that of uninfected disks, particularly with 1.0 and 10 ppm IAA. At these higher concentrations, the IAA apparently acted in conjunction with some product of bacteria, causing the increased inhibition. Usually disks inoculated with A. tumefaciens were stimulated only

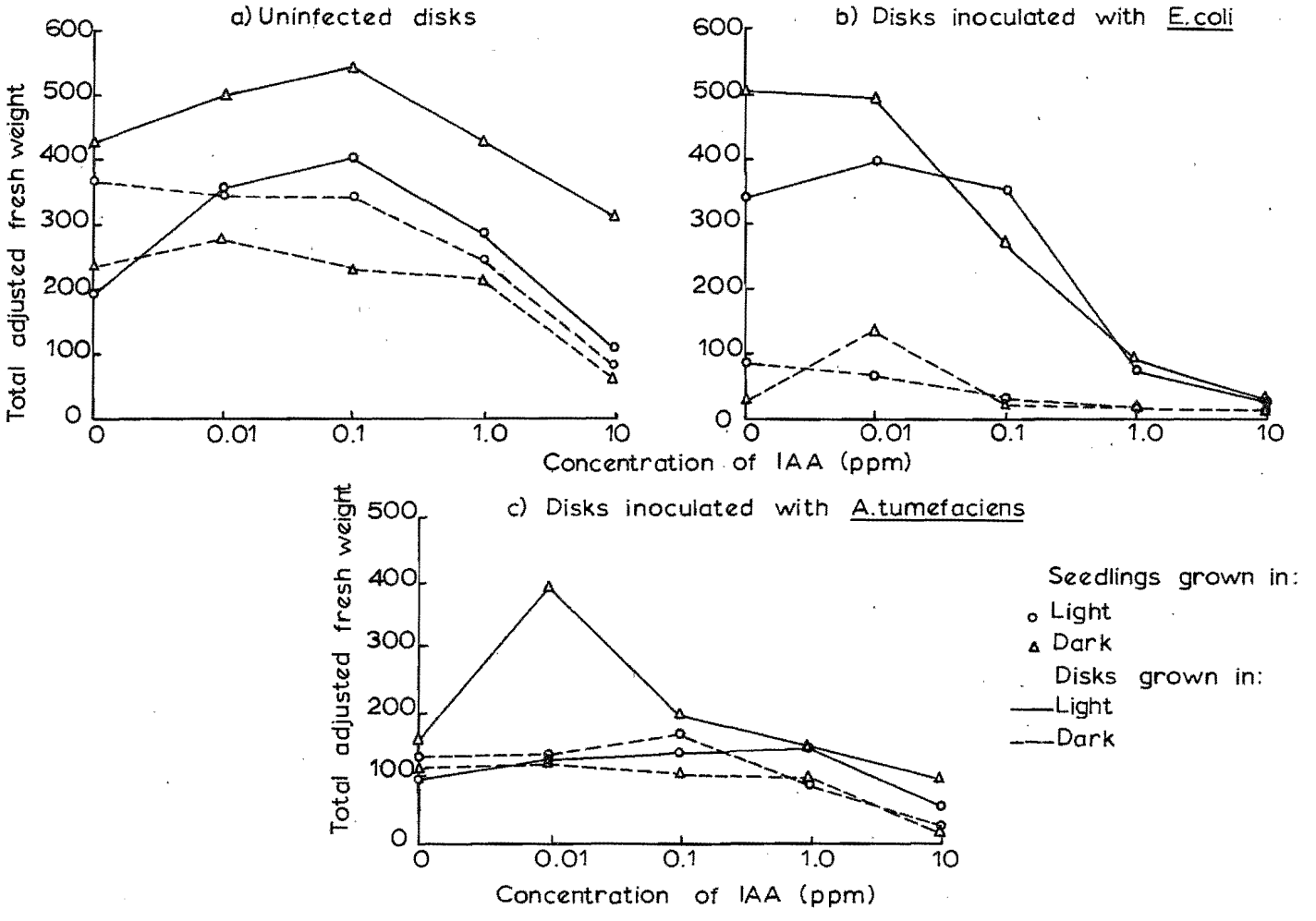


FIGURE 5.11 INTERACTION BETWEEN SEEDLING-LIGHT AND DISK-LIGHT TREATMENTS FOR EACH BACTERIAL TREATMENT

a little by increasing the concentration of IAA in the medium to 0.1 or 1.0 ppm, while growth was comparatively inhibited with 10 ppm IAA. With this bacterium, an interaction occurred between the seedling-light treatment and IAA at a concentration of 0.01 ppm; this will be discussed in the following section. With concentrations of IAA up to 0.1 ppm, A. tumefaciens-infected disks were generally the lightest of the three bacterial treatments, but with 1.0 and 10 ppm IAA, E. coli-infected disks were more inhibited.

Disks grown in the dark (Figure 5.10c and d) were not as heavy as those grown in the light, and the same bacterial treatments showed quite different trends. Growth of uninfected disks was not stimulated by low concentrations of IAA (0.01 and 0.1 ppm), while it was distinctly inhibited with higher concentrations. Morphological observations showed that these disks became more hyperhydric with lower concentrations of IAA than those grown in the light. Through this, root formation was suppressed and consequently dark-grown disks had smaller fresh weights (Figure 5.11a). Disks inoculated with E. coli were very much lighter than uninfected disks, when grown in the dark. The addition of IAA to media on which these disks were grown mainly had an inhibitory effect, although disks from dark-grown seedlings were stimulated with 0.01 ppm IAA. Again, this represents an interaction between seedling-light and IAA treatments which will be discussed in the following section. At all concentrations of IAA, disks infected with E. coli were the lightest of the three bacterial treatments. This inhibition of E. coli-inoculated disks in the dark was most marked when compared with similarly

infected disks grown in the light (Figure 5.11b). Disks inoculated with A. tumefaciens and grown in the dark, were stimulated only slightly by the low concentrations of IAA up to 0.1 ppm and were inhibited by the higher concentrations (Figure 5.11c). As in the light, the disks were much lighter than uninfected disks.

In the light, growth of disks affected by the different bacterial treatments varied greatly with the concentration of IAA from 0.01 to 1 ppm, but with 10 ppm growth was markedly inhibited in all cases, so that there were smaller differences between the treatments. Less variation occurred with inoculated disks grown in the dark, and no concentration of IAA differentially stimulated the growth of disks to any great extent.

5.14 Interactions between Seedling-light, Disk-light and Bacterial Treatments. (Figure 5.11)

An interaction between seedling-light and disk-light treatments occurred in disks three to six weeks old showing that in the light, disks prepared from dark-grown seedlings were heavier than those from seedlings grown in the light. This difference was generally present over the complete range of concentrations of IAA, for each bacterial treatment, but was most marked for uninfected disks. A smaller interaction was evident between dark-grown disks and their seedling-light treatment. In this case, in contrast to disks grown in the light, most disks from seedlings grown in the light were heavier than those from dark-grown seedlings over the range of IAA concentrations. Again, the greatest difference between the two occurred with uninfected disks.

From the gross morphology of uninfected disks grown in the light on media with no IAA or 0.01 ppm IAA, it was observed that disks prepared from seedlings grown in the dark formed more roots than disks from light-grown seedlings. No differences in morphology were noted for uninfected disks grown with higher concentrations of IAA. However, from Figure 5.11 it may be seen that a constant difference between uninfected disks from dark and light-grown seedlings exists over the entire range of IAA concentration. While increased rooting certainly contributed to this effect at low concentrations of IAA, no explanation can be made for this difference in growth at higher concentrations. A difference also existed between E. coli-inoculated disks grown with different concentrations of IAA, when prepared from seedlings receiving different light treatments. This was particularly marked when no IAA was present in the medium. Morphological observations showed that disks from dark-grown seedlings formed more roots than those from seedlings grown in the light. No morphological differences were noted between infected disks grown on medium containing 0.01 ppm IAA, and the differences in weight were smaller, decreasing even further with higher concentrations of IAA. The weights of disks inoculated with A. tumefaciens and grown in the light were also influenced by the seedling-light treatment. The most marked difference was apparent between disks grown on medium containing 0.01 ppm IAA. In this case those grown from dark-grown seedlings produced more roots and were correspondingly heavier.

For uninfected disks grown in the dark on medium with no

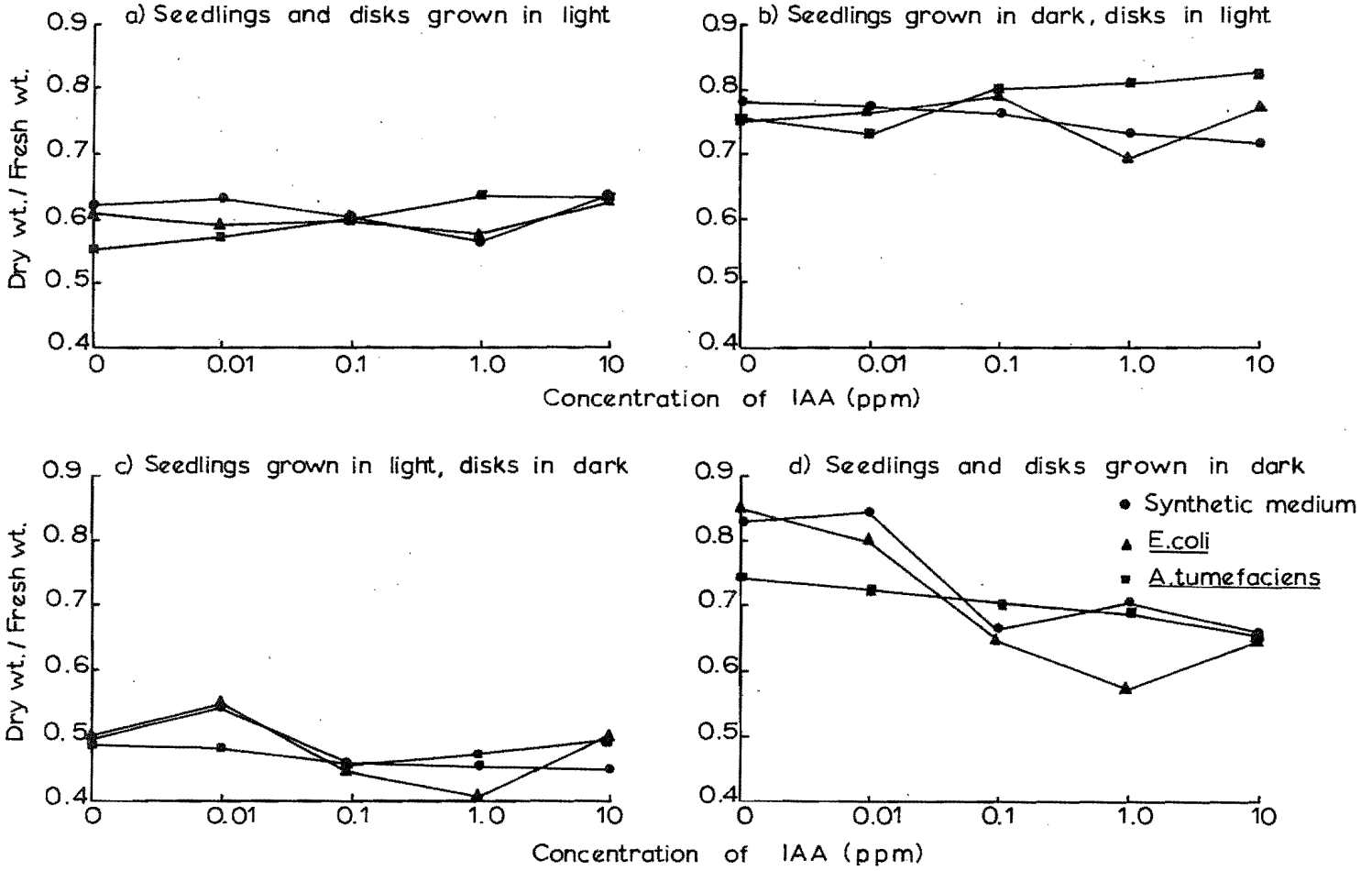


FIGURE 5.12 RATIO OF DRY WEIGHT / FRESH WEIGHT FOR EACH BACTERIAL TREATMENT GROUPED ACCORDING TO SEEDLING-LIGHT AND DISK-LIGHT TREATMENTS

added IAA, those prepared from dark-grown seedlings formed more roots, but it is apparent from the fresh weights that disks from light-grown seedlings must have been stimulated to more total growth. The weights of E. coli-inoculated disks prepared from seedlings grown in different light conditions diverged sharply when grown in the dark on medium containing 0.01 ppm IAA. In this case, disks from dark-grown seedlings were heavier than those from seedlings grown in the light, and morphological observations showed that the heavier disks had formed more roots. At higher concentrations of IAA, there was little detectable difference between the weights of these disks. The differences between A. tumefaciens-infected disks grown in the dark from seedlings grown in light and dark were not as large as those for light-grown disks.

Seedling-light treatment had less influence on E. coli and A. tumefaciens-inoculated disks grown in the dark than on those grown in the light, and had most influence on uninfected disks grown in both light conditions.

5.15 Analysis of the Dry Weight to Fresh Weight Ratios.

The ratios of dry weight to fresh weight of each treatment were compared to determine whether there were any differences among them in water uptake and cell division (Figures 5.12 and 5.13 and Table 8, Appendix III). It was immediately apparent that the greatest difference existed between seedling-light and disk-light treatments, rather than among the bacterial treatments as one would have expected. Dark-grown disks from seedlings

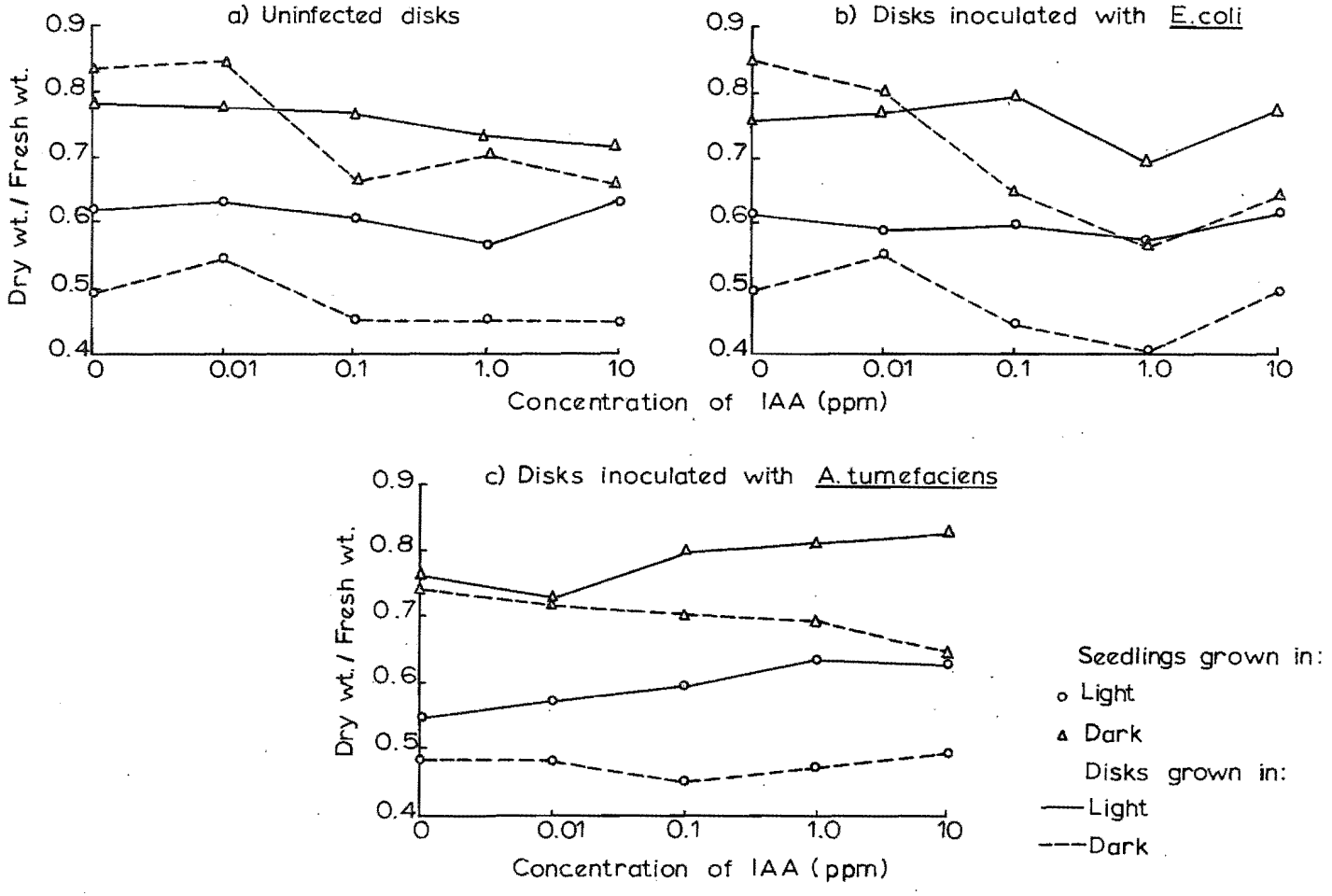


FIGURE 5.13 RATIO OF DRY WEIGHT/ FRESH WEIGHT FOR SEEDLING-LIGHT AND DISK-LIGHT TREATMENTS GROUPED ACCORDING TO BACTERIAL TREATMENTS

grown in the light showed the most water uptake of all the treatments, although with this combination there were variations with bacterial treatments and IAA concentration. Light-grown disks from seedlings grown in the dark generally showed the least water uptake of all the treatments, and again fluctuations occurred with bacterial and IAA treatments. Between these two sets of ratios lay those of light-grown disks from seedlings grown in the light, and dark-grown disks from seedlings grown in the dark. When seedlings and disks were both grown in the light, the ratios varied the least with change in concentration of IAA and bacterial treatment. Dark-grown disks from seedlings grown in the dark varied the most with increase in concentration of IAA. With no IAA, or concentrations of 0.01 ppm, disks showed more water uptake than those grown with higher concentrations of IAA, particularly in E. coli-inoculated disks.

When the dry weight to fresh weight ratios for each bacterial treatment were compared (Figure 5.13), it was found that for dark-grown disks prepared from seedlings grown in the light or the dark, the ratios were similar with each concentration of IAA. The same effect was observed for light-grown disks. In both the light and the dark, disks from seedlings grown in the light had a lower dry weight to fresh weight ratio than those from seedlings grown in the dark.

No explanation can be advanced for the differences apparent in Figures 5.12 and 5.13 in dry weight to fresh weight ratios for disks prepared from seedlings receiving different light treatments. The initial dry weight to fresh weight ratios of disks prepared

from seedlings grown in the light were higher than those from dark-grown seedlings showing that more water uptake had occurred in the latter seedlings. This is in accord with observations of the histology of etiolated tissues summarized by Sinnott (1960), and also with the descriptions in Chapter 9 of dark-grown etiolated tissues which had larger parenchyma cells and less secondary thickening in the vascular tissues than disks prepared from seedlings grown in the light. The ratios for the disks after six weeks of treatment however bore no relationship to the initial ratios, and were in fact the complete reverse; the disks from light-grown seedlings showed more water uptake than those from seedlings grown in the dark. Thus the light treatment of the disks following inoculation was important in determining the dry weight to fresh weight ratio. However some disks grown in the dark had a higher ratio than light-grown disks which is contrary to the observations for the younger tissues. The light treatment did not alter the dry weight to fresh weight ratios uniformly and these ratios cannot therefore be explained by considerations either of etiolation phenomena, or of different rates of carbohydrate production in the different light conditions. The explanation may lie in the effects of light together with carbohydrate formation on the auxin metabolism of the tissues. With the differently treated seedlings the widely varying ratios may have arisen through the different effects of disk-light treatment on the production of auxins.

In the light, the dry weight to fresh weight ratio of uninfected disks decreased gradually with increasing IAA concentration,

showing that the tissues gradually became more hyperhydric. In the dark, this decrease was most marked when the concentration of IAA was increased from 0.01 to 0.1 ppm. This showed that more water uptake had occurred (Figure 5.13a). Because of the great differences in fresh weights it would be expected that the ratios would differ for dark and light-grown disks inoculated with E. coli. In fact dark-grown disks inoculated with E. coli showed a steady increase in the amount of water uptake when grown on media containing increasing concentrations of IAA to 1 ppm, but less water uptake occurred with 10 ppm IAA. These dark-grown disks grew least of all the bacterial treatments, and it would appear that with increasing concentration of IAA, most of the increase in fresh weight was due to water uptake (Figure 5.13b). The dry weight to fresh weight ratios of E. coli-inoculated disks grown in the light were affected only a little by increasing concentrations of IAA, confirming the morphological observations that these tissues did not expand by water uptake alone but by associated cell division.

Disks inoculated with A. tumefaciens reacted quite differently from those receiving the other bacterial treatments. In most cases increases in the concentration of IAA did not affect the dry weight to fresh weight ratio to any great extent. Usually the tissues grown in the light showed slightly decreased water uptake with increasing concentrations of IAA, while dark-grown disks became hyperhydric but not to the same extent as in the other bacterial treatments (Figure 5.13c).

5.16 Discussion of the Effects of the Various Treatments on Growth of the Disks.

a) Effects of Disk-Light Treatment. As in Experiments 4 and 5, it was found that different light treatments had a marked influence on the growth of hypocotyl disks, both infected and uninfected. In uninfected disks growth was stimulated in the light throughout the range of IAA concentrations used, and the disks formed many cells with thick walls when no IAA was present in the medium. In the dark the tissues were etiolated so that cells were larger with thinner walls and throughout the range of IAA concentrations there was less growth (see Chapter 9). The light treatment also influenced the number of roots formed in uninfected disks, so that more were produced in the dark than in the light when no IAA or low concentrations of IAA were used.

Besides its action on photosynthesis, light affects many processes in plant growth and development including growth substance metabolism. Since auxins at different concentrations are closely associated with the initiation of roots and their continued growth, it is likely that any effect of light which alters levels of auxin in the tissues will also alter the potentialities for root formation. In several cases it has been reported that darkness has a definite stimulatory effect on root initiation and growth (Nitsch 1963, Fries 1960). In lupin seedlings, Fries found that kinetin and adenine could not promote root growth in the light and suggested that light may have caused a change in sensitivity of the roots to these substances. Gautheret (1961) grew tissues from two varieties of Jerusalem

artichoke and found that in one, light promoted the formation of roots while in the other, root formation was inhibited in the light and promoted in darkness. In uninfected sunflower hypocotyl disks it is apparent that light has an inhibitory effect on the formation of roots and their subsequent growth.

In disks inoculated with E. coli, different light conditions had a disproportionately large effect on growth. When no IAA or low concentrations were present in the medium, growth of E. coli-inoculated disks in the light was stimulated above that of uninfected disks but was comparatively inhibited with higher concentrations of IAA. In the dark growth of the infected disks was definitely inhibited compared with that of uninfected disks at all concentrations of IAA. To the writer's knowledge there have been no reports of plants responding in different ways to bacterial treatment when grown under different light conditions. However certain fungal diseases, such as Fusarium wilt of tomato, occur only in plants with a low sugar content (Foster and Walker 1947). In this case, plants grown in low light intensity are more susceptible to the disease than those grown in the high light. The interaction of E. coli with light conditions in causing proliferation or inhibition of growth in sunflower tissues may be connected with the different metabolism of the tissues when grown in the light or the dark. Evidence to support this is present in the report by Kuraishi and Muir (1964) that apical tissues of sunflower grown in the light and the dark produce different types of auxin. In the light the active compound had an Rf value of 0.1 to 0.2 and could not be identified with IAA or any of its

complexes in plant tissues. The Rf value of the auxin diffusing from shoot apices in the dark was between 0.7 and 0.8, and was comparable with that of synthetic IAA. It is possible that in sunflower hypocotyl disks, E. coli reacts with these auxins in different ways, producing the characteristic results observed. When cultures of E. coli were grown in the light and the dark there were no differences in their appearance although metabolic processes, which were not analysed, may have been altered by the light conditions. However, no reference could be found describing such changes.

Disks inoculated with A. tumefaciens and grown under different light conditions behaved in a reverse manner to E. coli-infected disks. With only one exception (that of inoculated tissues grown on medium containing 0.01 ppm IAA) there was only a slight variation between infected disks grown in different light conditions over the entire range of IAA concentrations. De Capite (1955) showed that although bacteria-free sunflower crown gall tissues are not green, their growth is increased in the light with increase in temperature of the tissues above 26°C. He concluded that this stimulatory effect of light was on some process other than photosynthesis. In the present experiment, however, no such stimulation was observed. This is in agreement with the finding of de Ropp (1948a) that light did not stimulate growth of crown galls in sunflower plants, and with that of Klein and Tenenbaum (1955) who showed that crown gall formation in secondary phloem of carrot was not influenced by the light conditions under which the tissues were grown.

These differing reports are probably due to the different methods of investigation used, as well as to differing plant tissues.

b) Effects of Seedling-Light Treatment. Besides the direct effects of light on the growth of hypocotyl disks, the light treatment of the seedlings from which the disks were prepared also influenced the growth form of the disks, particularly in their ability to produce roots. In uninfected disks prepared from seedlings grown in the light, root formation was inhibited compared with that in disks from dark-grown seedlings on medium without added IAA, regardless of whether the disks were grown in the light or the dark. This effect was also evident when uninfected disks were grown in the light on medium containing 0.01 ppm IAA, but with higher concentrations there was no apparent effect of seedling-light treatment on root formation.

Disks inoculated with E. coli and grown without added IAA reacted similarly to uninfected disks in the light in their response to the seedling-light treatment. Although the E. coli-inoculated disks produced more roots than uninfected disks under both seedling-light treatments, those prepared from dark-grown seedlings formed even more roots than those from seedlings grown in the light. In these infected disks the bacteria did not entirely overcome the inhibition of rooting caused by the seedling-light treatment. On medium containing 0.01 ppm IAA, E. coli-infected disks grown in the dark showed an effect of seedling-light treatment not observed with any other treatment combination. Disks prepared from dark-grown seedlings proliferated much more than those from seedlings grown in the light. It is apparent

that with this concentration of IAA and the seedling-light treatment, the inhibition of growth in E. coli-inoculated disks in the dark was overcome.

Root formation was also affected by the light treatment of the seedlings in A. tumefaciens-inoculated disks grown on medium containing 0.01 ppm IAA. In this case, disks grown in the light from dark-grown seedlings produced more roots than those prepared from seedlings grown in the light. In all other instances, the growth of disks inoculated with A. tumefaciens was not markedly influenced by the light treatment the seedlings received. In this respect disks inoculated with A. tumefaciens differed from either uninfected or E. coli-inoculated disks.

It seems likely that as with light treatment of the hypocotyl disks, the increased root response of certain disks can be explained by the alteration of growth substance levels in the plant tissues, in this case at the time of preparation of the hypocotyl disks. It would appear that both seedling-light and disk-light treatments are effective in modifying the response of the hypocotyl disks to the bacteria with which they have been inoculated.

c) Effects of IAA Treatment. Bausor (1942) found that application of IAA to stems of tomato cuttings grown in the dark was not effective in inducing rooting or callusing unless a source of carbohydrate was present in the medium, when the cuttings reacted in exactly the same way as those grown in the light. In the present work sucrose was included in the medium. It was therefore assumed that there was no fundamental difference

in the way IAA acted on hypocotyl disks grown in the light or the dark, but that differences occurred because of variations in the levels of the natural growth substances present in the plant tissues and in the rate of uptake of IAA from the medium. With uninfected disks it was apparent that those grown in the dark became hyperhydric at lower concentrations of IAA than light-grown disks. It is possible that some degradation of the IAA present in the medium occurred when disks were grown in the light. De Ropp and Markley (1955) also noticed that the response of segments of sunflower hypocotyl to lower concentrations of auxin was reduced by light. There are conflicting reports as to whether light increases or decreases the amount of auxin produced in plant tissues, or the quantity taken up by the tissues from the medium (Kuraishi and Muir 1964, Wickson and Thimann 1960, Thimann and Wardlaw 1963). It seems likely that less IAA, both natural and supplied, was present in the hypocotyl disks grown in the light than in the dark, but the method by which this occurred was not ascertained.

The principal object of this experiment was to compare the growth of uninfected disks on media containing different concentrations of IAA with that of disks inoculated with E. coli. In the light, the growth curves of infected and uninfected disks were similar although the E. coli-inoculated disks were stimulated, and subsequently inhibited, by lower concentrations of IAA than uninfected disks (Figure 5.10a and b). When the graph of the adjusted fresh weights of E. coli-inoculated disks is transposed one place, it can be seen that this curve fits that for

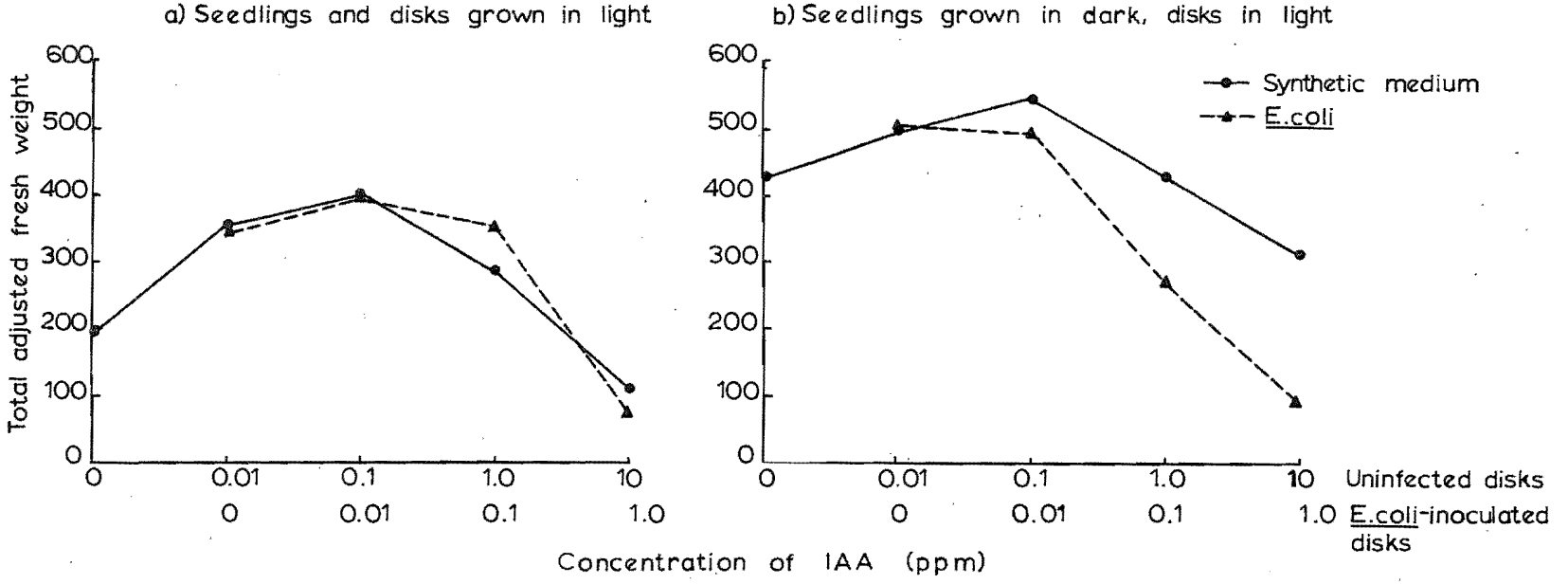


FIGURE 5.14 TOTAL ADJUSTED WEIGHTS OF E.COLI-INOCULATED DISKS COMPARED WITH THOSE OF UNINFECTED DISKS ASSUMING THAT BACTERIAL STIMULATION IS EQUIVALENT TO 0.01ppm IAA

uninfected disks, particularly when they had been prepared from seedlings grown in the light (Figure 5.14). When low concentrations of IAA were used, inoculation with E. coli had an effect on disks similar to the presence of 0.01 ppm IAA in the medium. With higher concentrations of IAA, the growth of E. coli-inoculated disks was inhibited comparatively more than that of uninfected disks, particularly when seedlings grown in the dark had been used. No similar comparisons could be made between E. coli-inoculated and uninfected disks grown in the dark. The weights of E. coli-inoculated disks grown without added IAA were similar to those of uninfected disks grown on medium containing 10 ppm IAA. It is most unlikely that the bacteria act in a similar way on the tissues in both the light and the dark when such large disparities in weight are evident.

The reactions of A. tumefaciens-infected disks to the presence of different concentrations of IAA in the medium were quite different from those of either E. coli- inoculated or uninfected disks. Although crown gall tissues are hyperauxinic (Kulescha and Gautheret 1948) it is apparent that additional IAA applied externally does not influence the reactions of the tissues to any great extent, except to cause inhibition at the highest concentration, 10 ppm (Hildebrandt and Riker 1947). This contrasts with the stimulation and inhibition with increasing concentrations of IAA in uninfected and E. coli-inoculated disks. The effect of inoculation with A. tumefaciens is thus not a simple increase in the IAA content of the tissues.

5.17 Conclusions.

E. coli-inoculated tissues grown in the light respond with a similar weight increase to that of uninfected disks grown on medium containing low concentrations of IAA, although the form of proliferation of these tissues is different. This implies that the substance formed by the bacteria is not identical with IAA. Confirmation of this is provided by the inhibition of growth in dark-grown disks inoculated with E. coli. Growth of A. tumefaciens-inoculated disks shows no similarity with that of uninfected disks grown on medium containing IAA. Thus it is concluded that the action of E. coli on sunflower hypocotyl disks is quite different from that of A. tumefaciens but is similar, although not identical, with that of IAA when tissues are grown in the light.

CHAPTER SIX

ADDITIONAL EXPERIMENTS WITH DISKS

De Ropp and Markley (1955) reported that in sunflower hypocotyl segments 5 mm long, grown on media containing varying concentrations of IAA, the increase in fresh weight was greatest when the segments were grown with the radical end resting on the agar. In the present work a comparison was made of the growth of 1 mm hypocotyl disks placed radical or apical end in contact with the medium to determine whether these segments differed from the longer ones described by de Ropp and Markley.

It had been noted in previous experimental work that in certain hypocotyls some of the disks showed an unusual type of growth which was not seen in the majority of the disks. Disks were grown in close proximity in order to determine whether the way in which one disk reacted influenced those growing nearby.

In the initiation of crown galls, the bacteria must enter the plant through freshly wounded tissue. The proliferation induced by Bacillus megaterium was shown to be completely independent of wounding (Fallot 1964). An experiment was therefore designed to determine whether wounding plant tissues prior to inoculation influenced subsequent growth in the presence of Escherichia coli.

The following set of experiments then, was designed to test further the reactions of sunflower hypocotyl disks to inoculation with E. coli. The seeds used for these experiments were harvested from plants grown from the batch of seeds used in

Experiment 9.

EXPERIMENT 10. GROWTH OF HYPOCOTYL DISKS WITH THE BASAL OR APICAL END IN CONTACT WITH THE MEDIUM.

In the previous experiments (numbers 4,5 and 9), 1 mm thick hypocotyl disks were grown with the radical end in contact with the medium. It was assumed that most of the IAA diffused into these tissues and was not dependent on processes governing polar uptake in a basipetal direction. Studies of young seedlings have shown that auxin transport is mainly basipetal (Jacobs 1961), although in segments of seedlings grown on medium containing high concentrations of auxin (1000 ppm) transport from base to apex occurs (Went and White 1939). De Ropp (1947a, 1951c) carried out a series of experiments using segments of sunflower tissue grown on media containing various growth substances. In the first of these experiments (1947a) segments 3 mm long from the first internode were grown on media containing auxins so that the basal end rested on the medium. In a later experiment (1951c), segments of hypocotyl 5 mm long were grown with the apical end resting on the medium which contained IAA. No reason was given for this change in technique, but in 1955 a comparison was made between the growth of 5 mm segments of hypocotyl grown with either the basal or apical end in contact with the medium which contained various concentrations of auxin (de Ropp and Markley). Polar transport of auxin, measured by increased elongation of certain segments, was shown to occur from the apical to the basal end of the segments. The fresh and dry weights of the segments were

influenced in a different way, so that those grown with the basal end in contact with the medium were heavier than those grown with the basal end uppermost.

The present experiment was carried out to determine whether the transport of auxin from the medium influenced the fresh weights of 1 mm thick hypocotyl disks grown with either the basal or apical end resting on the medium.

6.1 Experimental Details.

Sterile seedlings were grown, both in the light and the dark, and disks were prepared from the hypocotyls. Half of these were placed apical end and half basal end in contact with de Ropp's medium which contained either no IAA, or concentrations of 0.01, 0.1 or 1.0 ppm IAA. Disks were inoculated with either sterile synthetic medium or a 24-hour culture of E. coli in synthetic medium, and were grown in the same light conditions as the seedlings from which they were derived. Ten replicates of each treatment were prepared. After four weeks' growth, fresh and dry weights of the disks were measured.

6.2 Results and Discussion.

The disks were similar in form to those described in Experiment 9, although a high proportion of E. coli-inoculated disks grown in the dark proliferated and produced long roots. The usual reaction of such disks, observed in previous experiments, was to grow only a little before death. As far as could be determined by inspection, there were no gross morphological differences between disks treated in the same way, and grown with either the apical or basal end in contact with the medium.

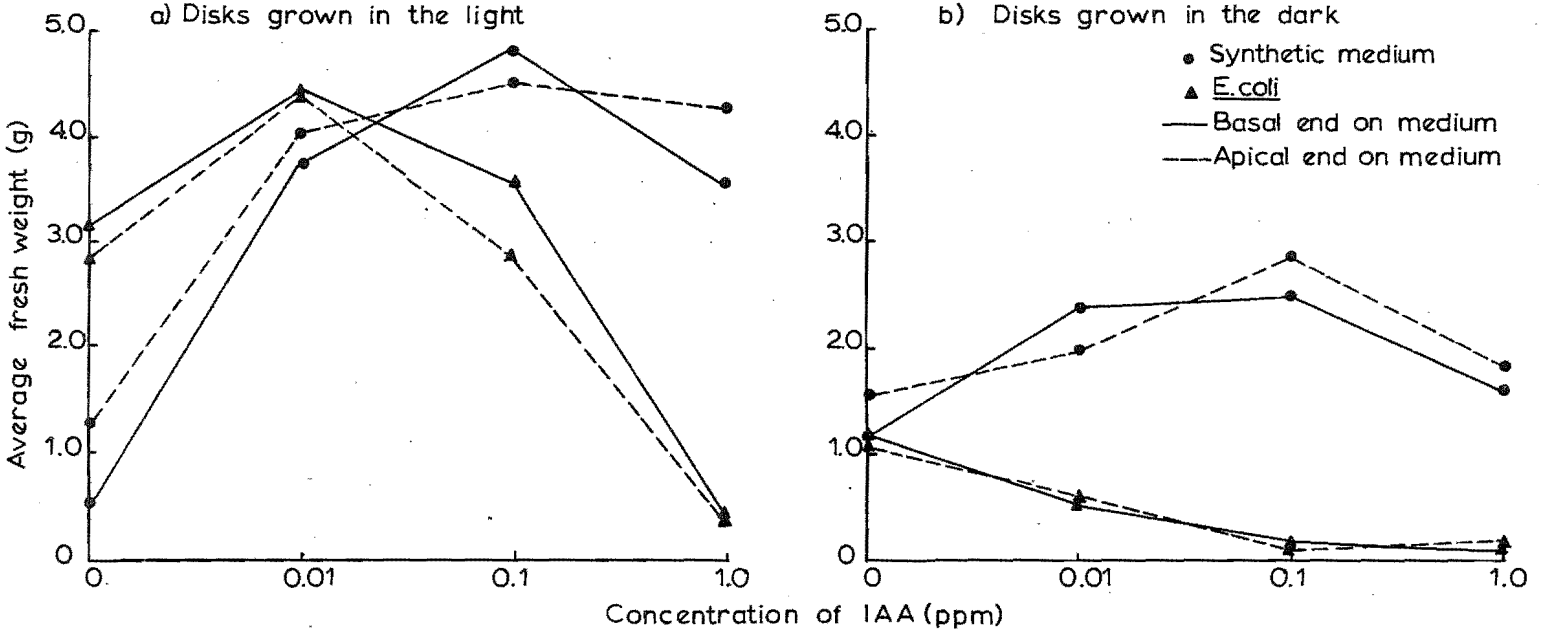


FIGURE 6.1 AVERAGE FRESH WEIGHTS OF DISKS GROWN FOR FOUR WEEKS WITH EITHER APICAL OR BASAL END RESTING ON THE MEDIUM UNDER DIFFERENT LIGHT CONDITIONS

The average fresh weights of disks treated in the same way, but grown with either the apical or basal end in contact with the medium were similar (Figure 6.1 and Table 1, Appendix IV). In two cases with uninfected light-grown disks (grown without IAA and with 1 ppm IAA), those with the apical end in contact with the medium were significantly heavier than those grown with the apical end uppermost (at the 10% and 5% levels respectively). In the dark, there was only one instance where a significant difference occurred between disks grown radical or apical end in contact with the medium. In contrast to the light-grown disks, uninfected disks grown with the radical end in contact with medium containing 0.01 ppm IAA, were significantly heavier (at the 2.5% level) than those grown with the apical end in contact with the medium. (See Appendix IV for details of significance test). Since there was no consistent difference in the weights of disks grown either radical or apical end in contact with the medium, it would appear that uptake of IAA was not necessarily polar, since it did not influence the final weights of the disks.

The results have shown that in general, IAA is reactive at the tissue surface in contact with the medium, whether it is morphologically the basal or apical end of the hypocotyl disk. Beal (1940), using thin sections of the second internode of bean seedlings, showed that when the segments were placed with either the apical or basal end on the nutrient containing IAA, swelling only occurred at the end in contact with the medium. The present observations of sunflower tissue growth are in agreement with those for bean segments. Segments of sunflower hypocotyl,

1 mm thick, appear to behave differently from the 5 mm thick segments described by de Ropp and Markley (1955). The differences in fresh weight between segments grown apical or basal end in contact with the medium were not found with 1 mm segments.

EXPERIMENT 11. DIFFERENT GROWTH RESPONSES OF SIMILARLY TREATED HYPOCOTYL DISKS

During the course of experimental work, it was observed that some disks prepared from the same hypocotyl, grew differently. Those taken from the upper part of the hypocotyl grew more in length and less in breadth, and produced fewer roots than those from lower down. In some flasks, disks inoculated with E. coli and grown in the dark, became necrotic while in identical conditions, others showed all disks proliferating. It was not known whether one disk influenced adjacent disks in the way they reacted, or whether this effect was due only to the genetic constitution of the plant. By growing in one flask all disks which had been sectioned from the same level in a number of hypocotyls, it could possibly be determined how much the response of the disks was due to the individual characteristics of each hypocotyl, and how much to the proximity of differently reacting disks.

6.3 Experimental Details.

Sterile seedlings were grown in the light or the dark and selected in groups of ten for the preparation of hypocotyl disks. The first disks from each of the ten hypocotyls were placed in

one flask, the second in another, until ten flasks were prepared, each containing ten disks sectioned from the same part of the hypocotyls. The exact hypocotyl and the position of each disk sectioned from it were therefore known. Each of these series was inoculated with either sterile synthetic medium, or with a 24-hour culture of E. coli. The disks were replaced in the same light conditions as the seedlings from which they had been prepared, and were harvested after four weeks' growth. There were four replicates of each bacterial and light treatment.

6.4 Results and Discussion.

In the light none of the uninfected disks taken from the top 4 mm of the hypocotyls produced roots. In one hypocotyl, disks sectioned from 6 to 10 mm below the apex formed roots, but this response must have been inherent in these tissues, since it was not transmitted to any of the other disks in the same flasks. In several hypocotyls, disks sectioned from 9 to 10 mm below the apex formed roots. This is in accord with de Ropp's (1951c) findings for the longer segments, where it was shown that those sectioned more than 4 mm below the apex produced more roots than those from nearer the apex.

In the dark, uninfected disks usually proliferated a little and some roots were formed. The number of disks forming roots in each flask increased with increase in the distance of the tissues from the top of the hypocotyl. It was found, however, that certain disks scattered throughout the flasks, expanded mainly by water uptake and were hyperhydric. The results showed that there was no stimulus from such disks influencing

more normal disks growing nearby. As with root formation in light-grown disks, it was concluded that this type of growth was an inherent characteristic of the hypocotyls from which the disks were prepared.

In the light it was found that in most cases the amount of bacterial-induced proliferation from the lower surface of E. coli-inoculated disks increased as the disks were sectioned from lower positions in the hypocotyls. Most disks formed roots although larger numbers were produced from disks sectioned from near the base of the hypocotyl. More roots were formed than from uninfected disks grown in the light.

Disks inoculated with E. coli and grown in the dark showed the same variability of reaction evident in the previous experiment. In most of the disks there was greater growth than in uninfected disks, but in a few cases, growth was inhibited. The inhibited disks came from the same hypocotyls throughout the series of flasks, and these did not appear to influence neighbouring proliferating tissues. Again it was apparent that the reaction of dark-grown disks to infection with E. coli was dependent on the reactivity of the tissues.

De Ropp (1951c) grew 5 mm long segments of sunflower hypocotyl in the dark, and found that no roots were formed from segments taken from nearest the cotyledons, but an increasing number of segments formed roots with increasing distance from the apex. The present experiment confirmed de Ropp's findings.

The main aim in this experiment was to determine whether there were any interactions between neighbouring disks in the

same flask. The results show that there were no such interactions. Hypocotyl disks possess a potentiality to develop in a particular way, and this seems to be fulfilled independently of any influence from neighbouring disks.

EXPERIMENT 12. ASSOCIATION OF WOUNDING AND INOCULATION IN DETERMINING PROLIFERATION.

It has been found that the potential of plants to form crown galls varies with the time the bacteria are placed in contact with the plant after wounding. De Ropp (1948a) placed A. tumefaciens on fragments of sunflower stem tissue at various intervals after it had been excised. Most fragments formed tumours when the bacteria were applied immediately or three days after tissue isolation. Thirteen out of eighteen formed tumours when the bacteria were applied after a week, and none were formed when A. tumefaciens was put on tissues two weeks after their isolation. De Ropp concluded that the tissues were still able to react after a week. Braun (1947a), in a similar experiment, found that a high percentage of tumours developed on periwinkle plants that had been wounded for three days or less, before the bacteria were introduced. Usually a far smaller number developed on plants wounded four days prior to inoculation, and none were formed at five days. Further to this, Braun (1952) found that the plant cells required a time of conditioning after wounding, before tumours would develop. This conditioning process increased gradually with time after wounding and reached a maximum between the second and third day (60 hours), declining again until no

tumours were formed when bacteria were applied five days after wounding. In the tomato, Braun (1954) reported that wounded tissues remained conditioned up to two weeks following wounding. To determine whether E. coli, like A. tumefaciens, was effective in stimulating tissue proliferation only in conjunction with wounding, bacteria were introduced on to sunflower hypocotyl disks at twelve-hourly intervals up to five days after the disks had been prepared.

6.5 Experimental Details.

Disks were prepared from light and dark-grown sterile seedlings, and were returned to these light conditions after treatment. With both light and dark-grown disks, ten replicates were left uninoculated, and the remainder were inoculated with a 24-hour culture of E. coli at 12-hourly intervals from 0 to 120 hours after their preparation. This time range was the same as that used by Braun (1952). An "Agla" micrometer hypodermic syringe calibrated to produce droplets 10 to 11 microlitres in size, was used to place the bacterial suspension on the surface of the tissues without damaging the exposed plant cells. Ten replicates of each treatment were prepared. The disks were harvested four weeks after they had been sectioned from seedlings.

6.6 Results and Discussion.

Uninfected disks grown in the light proliferated only a little and usually no roots were formed. Disks inoculated with E. coli immediately or 12 hours after wounding, and grown in the light, produced a large amount of proliferation from the

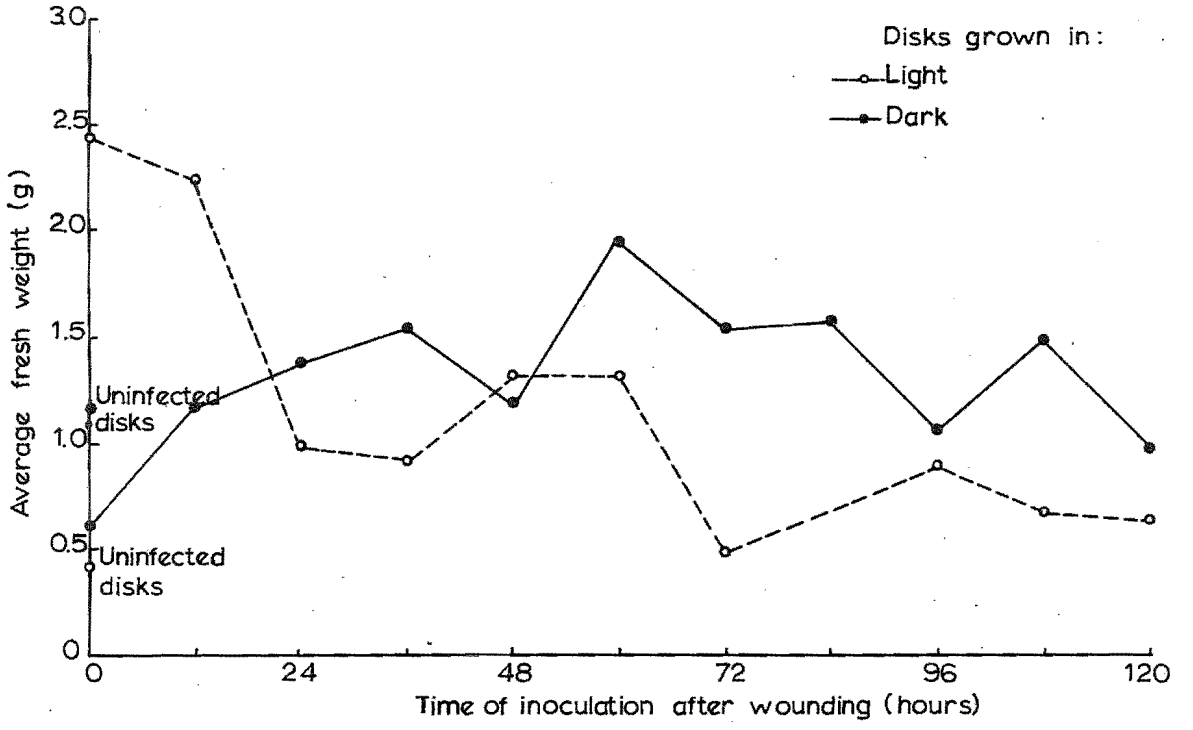


FIGURE 6.2 AVERAGE FRESH WEIGHTS OF DISKS INOCULATED AT DIFFERENT TIMES AFTER WOUNDING

lower surface. Most disks formed a number of long roots. With increasing time between preparation of the disks and their inoculation, fewer of the disks proliferated as markedly as those inoculated immediately. More roots were formed than in uninfected disks, but they were not as numerous as in disks inoculated immediately.

Changes in the average fresh weights of light-grown disks corresponded with changes in the amount of proliferation and root formation (Figure 6.2 and Table 2 Appendix IV). Disks inoculated immediately and twelve hours after wounding were very much heavier than uninfected disks. Although there were some fluctuations, the general trend was of a decrease in weight of the disks with increase in time between wounding and inoculation. However, even disks inoculated 120 hours after wounding, were heavier than the uninfected controls. Statistical analysis showed that only those disks to which the bacteria were applied immediately, 12, 48 and 60 hours after wounding were significantly heavier than uninfected disks (Appendix IV). In the light, it appeared that sunflower hypocotyl disks remained reactive to E. coli-inoculation up to 60 hours after wounding, but after this there was no significant increase in proliferation. Wounding therefore appears to be implicated in the reaction of the tissues to E. coli.

Inoculated disks grown in the dark did not react in the same manner as light-grown disks. When disks were inoculated with E. coli as soon as they had been prepared, in eight out of the ten flasks they expanded only a little, and grew less than

uninfected disks. In the remaining two flasks, the disks proliferated more from the lower surface than uninfected disks and formed many roots. Most disks inoculated 12 to 120 hours after preparation proliferated from the lower surface and formed long roots. The greatest amount of proliferation occurred in disks inoculated 60 hours after wounding.

The average weights of most disks inoculated with E. coli were higher than those of the uninfected disks, independent of the time the bacteria were applied after wounding (Figure 6.2). Disks increased in weight with increase in the time between wounding and inoculation up to 60 hours, and decreased after this. The average weight of disks inoculated immediately after wounding was similar to that of uninfected disks because growth in some cases was inhibited, and in others, increased, over that of uninfected disks.

In Experiments 10 and 11, the average fresh weight of the uninfected disks grown in the dark was 1.1596 g, but in the present experiment the average fresh weight of dark-grown disks was only 0.6080 g. These disks had not been inoculated with sterile synthetic medium, while in all other work, this inoculum had been applied. It would appear that the synthetic medium, by itself, may have had some stimulatory effect on dark-grown disks, either through increased supply of nutrients to the tissues, or because of the moister environment it provided for the disks. This effect was not evident in light-grown disks. The average fresh weights of dark-grown E. coli-infected disks, inoculated at various times after wounding, were compared with

the average weight of uninfected disks, found from Experiments 10 and 11. This was thought to be a truer comparison than that between infected and uninoculated disks.

Disks inoculated with E. coli immediately following preparation were significantly lighter than uninfected disks, (using values from Experiments 10 and 11), and only those inoculated 60 and 72 hours after wounding were significantly heavier than the uninfected disks. (Appendix IV). The inhibitory effect of the bacteria on growth was therefore most evident in disks inoculated immediately after wounding. This was the time at which most stimulation of light-grown disks occurred. With increasing time between wounding and inoculation, the inhibition of dark-grown disks decreased, while another factor causing stimulation of growth was increasingly expressed. This latter factor reached its maximum 60 hours after wounding.

In both light and dark, the time E. coli was introduced onto disks after preparation influenced the extent of response by the tissues. In both cases growth did not approach the levels of growth of uninfected disks until 72 hours after wounding. In the light, most stimulation occurred when E. coli was applied immediately and 12 hours after wounding, while in the dark most inhibition of growth occurred at this time. The reaction of dark-grown tissues was complicated by the stimulating action of the bacteria when applied at 60 and 72 hours after wounding. Wounding influences the reaction of tissues to inoculation with E. coli, so that the greatest stimulation or inhibition, depending on conditions of growth,

occurs when the bacteria are introduced into the wound immediately it has been made. With the return of the tissues to normal growth 60 to 72 hours after wounding, the bacteria are ineffective in stimulating the tissue. Wounding appears to play a different role in E. coli-infected tissues than in those inoculated with A. tumefaciens. Maximum response is obtained only if E. coli is applied immediately after wounding, while with A. tumefaciens the largest galls are formed when the bacteria are applied from 0 to 60 hours after the tissues have been wounded, and it is only after this time that gall formation decreases. The effect of wounding on the subsequent growth of E. coli-inoculated disks is also quite different from that in tissues inoculated with Bacillus megaterium. With this bacterium Fallot (1964) found that wounding had no effect on the subsequent development of inoculated tissues, so that the same growth was obtained when the bacteria were applied immediately, eight or 30 days after wounding.

EXPERIMENT 13. COMPARISON OF THE POWER TO INDUCE PROLIFERATION IN DIFFERENT STRAINS OF E.COLI

In the series of experiments (10-12) described in this chapter, the sunflower seedlings used were obtained from a recent seed harvest and differed by a generation from those used for previous experiments. It was found that with these tissues, the growth in the dark of E. coli-inoculated disks was different from that in Experiments 4, 5 and 9. Instead of little tissue growth, a large amount of proliferation occurred. The same

strain of E. coli was used throughout, but it was possible that it may have changed in character during the experimental period as the result of frequent sub-culturing. It is a well-known characteristic of bacteria that such changes may occur in the course of their culture. A culture of the strain of E. coli was kept separate from that being used throughout the work, and was only sub-cultured four times during the period of experimentation. A comparison was made between disks inoculated with the two cultures of E. coli to determine whether a change had occurred in the bacteria. The hypocotyl disks were prepared from seeds of recent origin, and from those one generation older, since the characteristics of the seedlings may have changed and thus altered the response to the bacteria.

6.7 Experimental Details.

Twenty seeds from each of the two harvests were sterilized and grown in the dark. Disks prepared from the seedlings were inoculated with either the culture of E. coli which had been sub-cultured infrequently (strain I) or with the culture used throughout the experimental work (strain II). There were ten flasks of each combination of bacterial strain and seed harvest. The inoculated disks were grown in the dark and harvested after four weeks.

6.8 Results and Discussion.

Since seedlings from two sources were used, an F-test was made on the initial weights of the hypocotyl disks. Disks derived from seeds of recent origin were slightly heavier than those from the previous season's harvest, but there was no

significant difference between them (Table 3 Appendix IV).

With both strains of E. coli some of the hypocotyl disks proliferated from the lower surface and produced long roots, while others grew only a little before becoming necrotic. There was no difference between the two strains in the nature of the morphological response produced, but in those inoculated with strain I, more disks showed only a little growth than in those inoculated with strain II. An analysis of variance of the fresh weights showed that there were no significant differences among the weights of differently treated disks (Table 4 Appendix IV). In this experiment the weights of the disks at harvest were only half the weight of comparable disks in Experiments 10 and 11. In Experiment 12, prepared only a little time after the two previous experiments, disks inoculated immediately had similar weights to those recorded in the present experiment.

It would appear that a slight change occurred in the characteristics of the strain of E. coli being used, so that different proportions of hypocotyl disks reacted by proliferating and forming roots, or by dying, when grown in the dark. This was manifest for only two experiments and gradually disappeared in subsequent work. However, this change has not influenced the main results of this work since it did not occur in critical experiments. It serves to emphasize that the results obtained are dependent on the particular strain of bacterium being used.

6.9 Discussion of the Additional Experiments with Disks.

These experiments clarified certain facts about the growth of hypocotyl disks and the action of *E. coli* on them.

The lack of consistent differences in the growth of disks placed apical or basal end in contact with media containing IAA was contrary to de Ropp and Markley's (1955) report that the growth of longer segments of sunflower hypocotyl was dependent on their orientation on similar media. The size of the disks was therefore important in determining the growth of the tissues when IAA was present.

The differing reactions of adjacent disks to the same inoculum was apparent throughout the work but it has been shown to be due more to the inherent variability of the plant material than to a changing effect of the bacterial treatment.

The most important results obtained in this chapter were from the experiment describing the growth of disks inoculated at different times following wounding. That *E. coli* produced most stimulation or inhibition of the tissues, depending on light conditions, when applied within twelve hours of wounding differentiated still further the action of this bacterium from that of either *A. tumefaciens* or *B. megaterium*.

C H A P T E R S E V E N

PITH TISSUE CULTURE

Some tissues require both cell enlargement and cell division factors for their continued normal growth in culture. Tobacco pith for example requires both of these, but the cell division factor is replaced when adjacent vascular tissues are present in the fragments (Jablonski and Skoog 1954). It is apparent that the type of growth shown by tissues is strongly influenced by the tissue composition, since certain cell-types are able to produce growth substances which can alter the course of growth of adjacent cells. By using homogeneous tissues, such effects can be eliminated and the direct reactions of bacteria or growth substances on the tissues can be studied.

The experimental work described in Chapters 3 to 6 was concerned with the reactions of whole sunflower plants, decapitated seedlings, and hypocotyl disks to inoculation with bacteria. In all cases the reactions of a heterogeneous collection of tissues were studied. In addition, all the material was genetically variable because of the outbreeding nature of sunflower plants which therefore produce variable seeds. A study of the effects of the bacteria on a homogeneous tissue was therefore undertaken as a supplement to this work. Sunflower pith was selected for this, and a clone of the tissue was established. By using material from a single clone, the biological variability encountered in earlier experiments was eliminated.

EXPERIMENT 14a. GROWTH OF INOCULATED PITH TISSUE ON DIFFERENT MEDIA

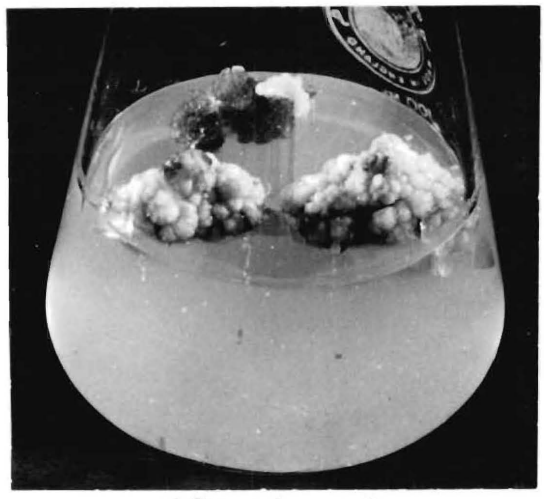
Sunflower pith tissue was grown on medium containing both cell enlargement and cell division factors, and the growth was compared with that of E. coli-inoculated tissues on a simple medium. The object was to determine whether the bacteria could replace any of the growth factors.

7.1 Experimental Details.

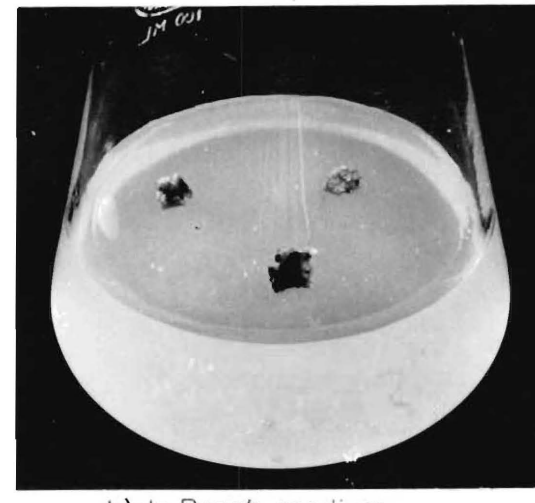
A clone of sunflower pith tissue grown on Skoog's medium containing IAA, kinetin and vitamin supplements, as described in Chapter 2, showed a rapid rate of growth, and was able to be subcultured every two to three weeks. The tissue was rather friable so that regular-shaped pieces were difficult to prepare. Pieces approximately 3 mm cubed were placed on either de Ropp's or Skoog's medium. Flasks containing 50 ml of medium, and sloped tubes containing 25 ml were used, three or two pieces of tissue being placed in the flasks or tubes respectively. The tissue pieces were inoculated with sterile synthetic medium, or with 24-hour cultures of E. coli or A. tumefaciens, and were grown in the light or the dark. Four replicates of each treatment were prepared except for tissues inoculated with A. tumefaciens when there was only one. Fresh weights of the tissues were recorded after four weeks of growth. Because of a failure of the drying oven, no dry weights were recorded.

7.2 Results and Discussion.

a) Growth of Pith Tissue in the Dark. Uninfected pieces of pith grown on Skoog's medium expanded and proliferated showing

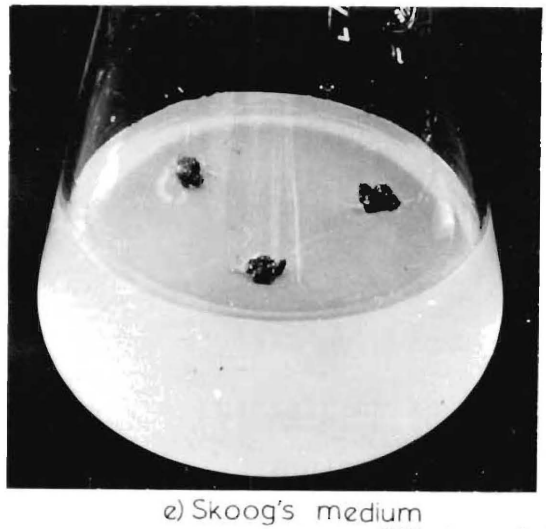


a) Skoog's medium

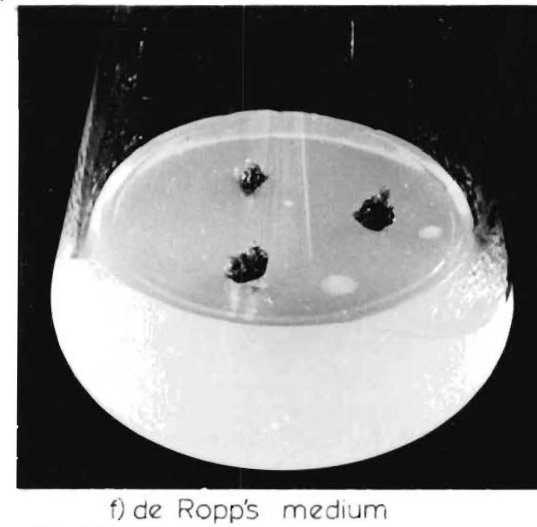


b) de Ropp's medium

Uninfected pith



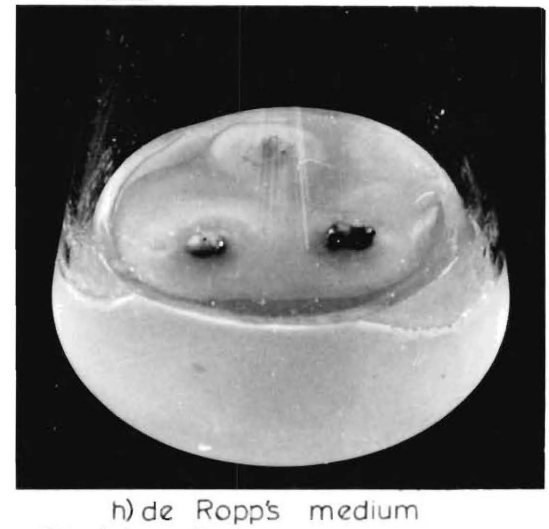
e) Skoog's medium



f) de Ropp's medium

Pith inoculated with E.coli

g) Skoog's medium



h) de Ropp's medium

Pith inoculated with A.tumefaciens

FIGURE 7.1 INOCULATED PITH TISSUE GROWN IN THE DARK ON SKOOG'S AND DE ROPP'S MEDIA (Mag. x 1)

a large amount of cell division. The tissue was usually white and firm, but in a few cases brown areas developed, where more water uptake had occurred (Figure 7.1a). In comparison, pith pieces similarly treated but grown on de Ropp's medium expanded only a little, although they were still alive (Figure 7.1b).

In previous experiments it was shown that although hypocotyl disks grew well on de Ropp's medium, the inclusion of 0.01, 0.1 and sometimes 1.0 ppm IAA in the medium enhanced growth. Pith tissue apparently has a higher requirement for IAA than the hypocotyl disks since Skoog's medium containing 2 ppm IAA supported good growth while only a little enlargement occurred when the pith was grown on de Ropp's medium containing no IAA.

The growth of E. coli-inoculated pith tissue on Skoog's medium was inhibited in comparison with that of uninfected tissues on the same medium. Tissues inoculated with E. coli and grown on both de Ropp's and Skoog's media became blackened very rapidly and showed no growth (Figure 7.1c, d).

Pith tissue inoculated with A. tumefaciens and grown on Skoog's medium in the dark proliferated from the upper surface where the bacteria were applied, but growth was not as great as in uninfected tissues (Figure 7.1e). On de Ropp's medium less proliferation occurred although cell division was still evident (Figure 7.1f). It has been noted previously that de Ropp's medium supports abundant growth of A. tumefaciens, and as with inoculated hypocotyl disks, some pieces of pith tissue became overwhelmed by the growth of the bacteria. On Skoog's medium

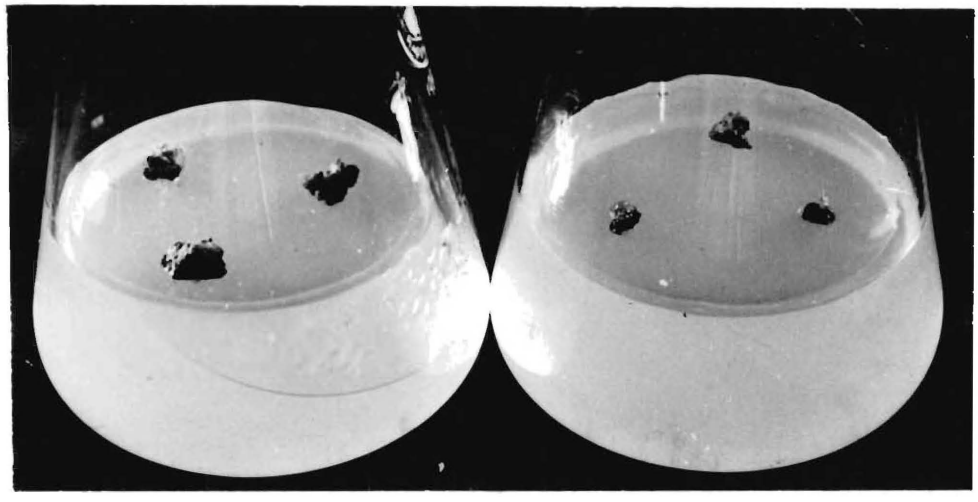


FIGURE 7.2 E.COLI-INOCULATED (Left) AND UNINFECTED PITH TISSUE GROWN IN THE LIGHT ON DE ROPP'S MEDIUM (Mag. x 1)

however, growth of A. tumefaciens was not supported to the same extent, so that the tissues could proliferate freely under the influence of the bacteria without the mechanical resistance to growth provided by the viscous fluid containing the bacteria.

b) Growth of Pith Tissue in the Light. Uninfected tissue pieces grown on Skoog's medium were even larger than similarly treated pieces grown in the dark. On de Ropp's medium, uninfected tissue pieces expanded only a little before becoming brown.

As in the dark, growth of E. coli-inoculated tissue on Skoog's medium was inhibited in comparison with that of uninfected pith, but on de Ropp's medium the pieces expanded more than uninfected tissues grown on the same medium (Figure 7.2).

A.tumefaciens-inoculated tissues proliferated more when grown on Skoog's medium than on de Ropp's.

c) Comparisons between differently-treated tissues. The average fresh weights of the tissue pieces are recorded in Figure 7.3 and Table 1 Appendix V. In both the light and the dark uninfected pieces grown on Skoog's medium were heavier than those inoculated with E. coli or A. tumefaciens. On de Ropp's medium, A. tumefaciens-infected pieces were heaviest, while tissues inoculated with E. coli were slightly heavier than uninfected pieces. The fresh weights of uninfected and E. coli-inoculated pieces grown on de Ropp's medium were compared, using the F-test (Appendix V). For tissues grown in the dark, those inoculated with E. coli were significantly heavier (at the 10% level) than uninfected tissues. Although infected tissues

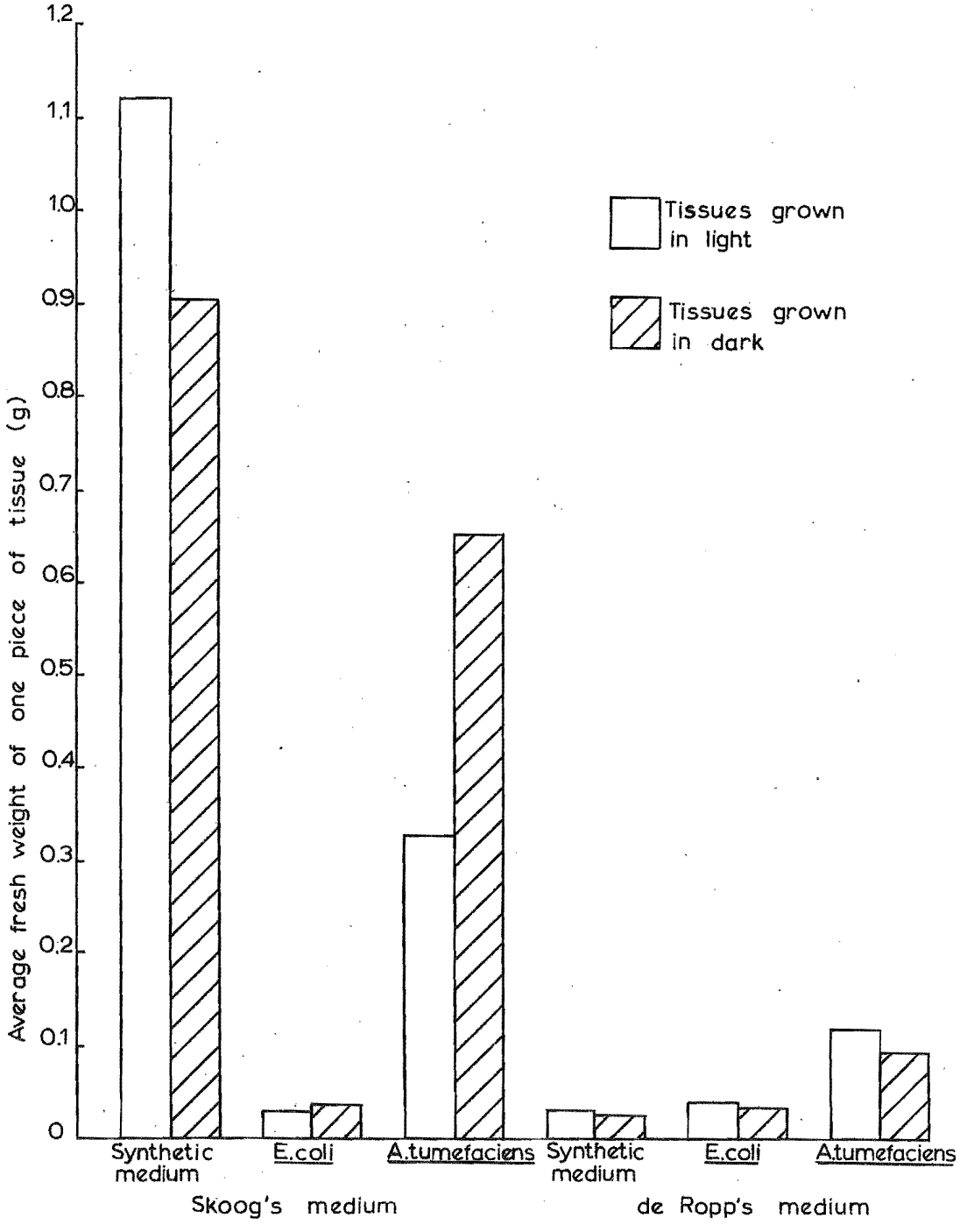


FIGURE 7.3 AVERAGE FRESH WEIGHTS OF DIFFERENTLY-TREATED PIECES OF PITH TISSUE AFTER FOUR WEEKS

grown in the light were also heavier than uninfected tissues, there was no significant difference between them. In both the light and the dark, tissues inoculated with E. coli were heavier when grown on de Ropp's medium than on Skoog's, although this difference was significant (at the 2.5% level), only for tissues grown in the light. Both in the light and in the dark, tissues inoculated with A. tumefaciens were stimulated more on Skoog's than on de Ropp's medium, the reverse of the reaction of E. coli-inoculated tissues. Inoculation of the pith tissue with bacteria apparently decreased the growth-promoting properties of Skoog's medium, more particularly when E. coli was used.

This limited test showed that sunflower pith tissue reacted in quite a different manner from 1 mm pieces of hypocotyl when inoculated with E. coli. For normal growth of the uninfected pith tissue, both kinetin and auxin were necessary. Inoculation with E. coli stimulated tissues grown on de Ropp's medium, but inhibited those on Skoog's medium. Inoculation with A. tumefaciens induced some growth both in the presence and the absence of the growth substances. On Skoog's medium these infected tissues were not inhibited as much as E. coli-inoculated pieces, while on de Ropp's medium infection with A. tumefaciens could not replace completely the requirements of the tissues for both auxin and kinetin. That more growth of A. tumefaciens-inoculated tissues occurred on the medium containing growth substances than on the simple medium is contrary to the finding of de Ropp (1947a) and Hildebrandt and Riker (1947). These workers found that growth of bacteria-free crown gall tissues was not

increased when auxins were included in the medium, and in most cases, inhibition of growth occurred. In the present case, growth may have decreased because of mechanical inhibition caused by the increased numbers of bacteria formed on tissues grown on de Ropp's medium.

Crown gall bacteria have been reported to inhibit the growth of carrot tissues cultured over some period of time (Gautheret 1959), and it is likely that the sunflower pith tissue behaved similarly in this respect.

EXPERIMENT 14b. FURTHER OBSERVATIONS OF GROWTH OF INOCULATED PITH.

A further test was carried out using 35 replicates of each treatment in order that a more highly significant difference could be measured between treated pieces of pith.

7.3 Experimental Details.

Pieces of tissue inoculated with E. coli were grown in the dark or the light on de Ropp's medium and on Skoog's medium. Uninfected tissues were also grown on de Ropp's medium in both light and dark conditions. In all cases, three pieces of pith tissue were placed in a flask containing 50 ml of medium, and after four weeks' growth, fresh and dry weights were recorded (Table 2 Appendix V). Comparisons between the fresh weights of the different treatments were made using the F-test (Appendix V).

7.4 Results and Discussion.

In the dark, uninfected tissue pieces were slightly heavier than those inoculated with E. coli when grown on de Ropp's

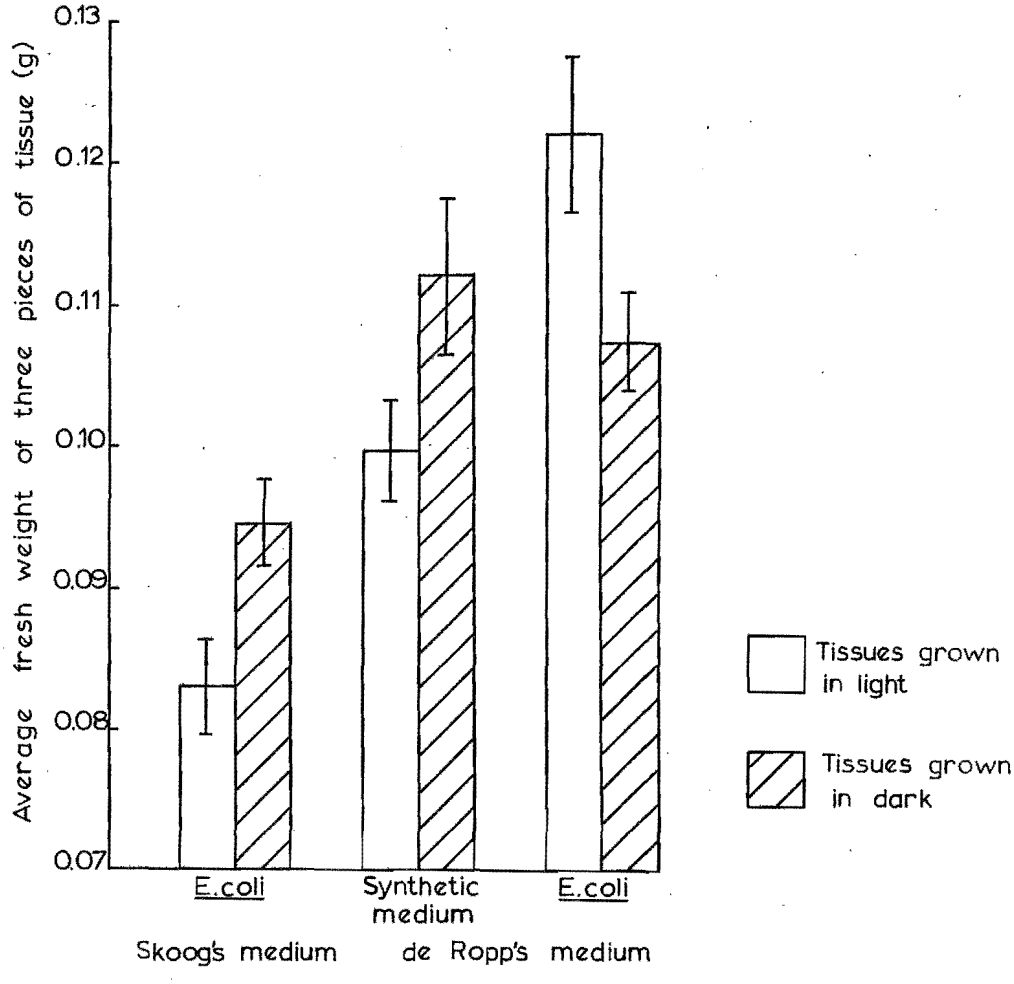


FIGURE 7.4 AVERAGE FRESH WEIGHTS AND STANDARD ERRORS OF DIFFERENTLY-TREATED PIECES OF PITH TISSUE AFTER FOUR WEEKS

medium. There was however, no significant difference between them. E. coli-inoculated tissues grown on Skoog's medium were not as heavy as those grown on de Ropp's medium, and again differences between them were not significant. In the light, E. coli-infected tissues grown on de Ropp's medium were significantly heavier (at the 0.5% level) than either uninfected pieces on the same medium, or infected tissues grown on Skoog's medium (Figure 7.4 and Table 2 Appendix V).

In the light, the weights of treated tissue pieces bore the same relationships to each other as were observed in Experiment 14a, although even greater differences were recorded. By contrast, dark-grown tissues reacted differently. The growth of E.coli-inoculated pith was inhibited compared with that of uninfected tissues, when grown on de Ropp's medium. Thus no definite trends of growth for tissues in the dark have emerged from this work. In the light, it would appear that E. coli is more effective in tissue stimulation when the pith tissue is grown on a simple medium, but is inhibitory to growth when auxin and kinetin are present.

On comparing the dry weight to fresh weight ratio for each treatment, it was found that less water uptake occurred in light-grown tissues than in those grown in the dark (Figure 7.5 and Table 3 Appendix V). In both light and dark, infected tissues grown on de Ropp's medium showed more water uptake than uninfected tissue. The largest amount of water uptake occurred in dark-grown E. coli-inoculated tissues on Skoog's medium. For E. coli-inoculated disks on both media it is

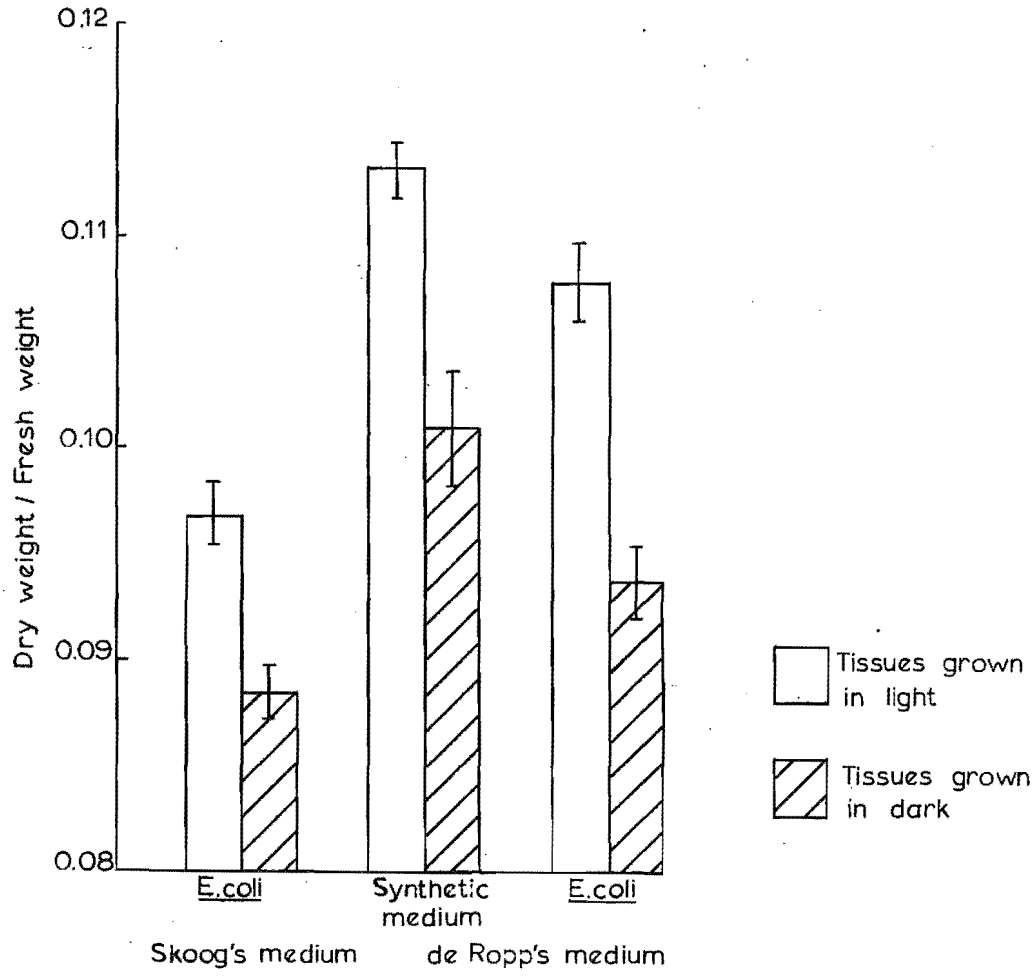


FIGURE 7.5 RATIO OF DRY WEIGHT / FRESH WEIGHT AND STANDARD ERRORS OF DIFFERENTLY-TREATED PIECES OF PITH TISSUE AFTER FOUR WEEKS

apparent from these results that most of the increase in fresh weight was due more to water uptake than to cell division.

7.5 Discussion of the Experiments with Pith Tissues.

There have been only a few reports of the influence of bacteria on the growth of tissues in culture. A. tumefaciens has been shown to stimulate the growth of parenchyma tissues from tubers of Jerusalem artichoke (Kulescha 1951), and pith parenchyma with associated xylem tissues of tobacco (Braun 1956). However, in carrot tissues, growth was inhibited after several passages of the tissues because the bacteria invaded the cells (Gautheret 1959).

Fallot (1964) has tested the action of numbers of bacteria on tissues from tubers of Jerusalem artichoke. Bacillus megaterium, Azotobacter chroococcum, Beijerinckia indica, Pseudomonas fluorescens and Sarcina lutea all stimulated growth, but this occurred in different parts of the explants according to the bacteria used. Bacillus subtilis and B. pumilus were without effect on this tissue. B. megaterium did not induce cell division in tobacco pith tissues. The reactions of bacteria-free crown gall tissues of Scorzonera, carrot and tobacco to inoculation with B. megaterium were also observed, and in all cases growth was completely stopped.

Volcani, Riker and Hildebrandt (1953) treated tissue cultures of carrot, periwinkle, potato and marigold with six different isolates of soft rot bacteria. In many cases the tissues were destroyed by the bacteria and in no case was growth stimulated.

From these results with tissue cultures it is apparent that the type of reaction manifest by the bacteria in the tissues is dependent on the tissues themselves. Thus Fallot obtained variable results when B. megaterium was applied to various types of tissues. From the present experimental work it is evident that pith tissue of sunflower is not stimulated to any great extent by E. coli since blackening of the tissues occurs shortly after inoculation, and that this tissue is much less reactive to this bacterium than the association of tissues comprising the hypocotyl. E. coli is effective in preventing the proliferation of the tissues on a medium containing growth substances, in contrast to the hypocotyl disks which proliferated when low concentrations of IAA were present. On a simple medium, E. coli stimulates increased water uptake by the pith tissues, although it is doubtful whether this is accompanied by cell division. For normal growth, sunflower pith tissue requires a number of growth substances including auxin, kinetin and vitamin supplements, and these requirements may be replaced only in small part by inoculation with E. coli. Since the bacteria produce some vitamins in culture (Burkholder and McVeigh 1942), these may have been responsible for the increased water uptake. A direct comparison between growth of the inoculated pith tissue on Skoog's medium, with and without the growth supplements, would be required however, before any definite suppositions could be made about the mode of action of E. coli.

While A. tumefaciens was able to induce proliferation of the pith tissue, it was apparent that the tissues were later completely covered by the bacteria and growth in some places ceased. It was of note that unlike E. coli, A. tumefaciens did not completely inhibit growth of the pith tissue on the medium containing growth substances. The tissue requirements for growth substances were partially replaced by A. tumefaciens. It is evident that E. coli and A. tumefaciens react quite differently on sunflower pith tissue.

CHAPTER EIGHT

HISTOLOGY OF ABNORMAL GROWTH FORMS

Tissue responses to growth substances, bacteria, viruses and genetic factors have been studied in a wide range of plants. Because of the complex nature of the plant body different agents may induce a similar response in a particular part of the plant, while in a single plant one agent may be responsible for completely different forms of growth in the root as compared with the shoot (Bloch, 1965). Proliferation is the most common reaction to such agents, but other responses such as the formation of roots or buds may occur. According to Gautheret (1959) these manifestations of growth are only possible if the tissue shows some tendency to form such organs; the growth-inciting agents will not stimulate the formation of organs in tissues which are not able to produce them spontaneously. However, all except highly specialized cells can dedifferentiate and form organs again. Skoog and Miller (1957) have shown that it is possible to induce both bud and root formation in tobacco pith cultures with kinetin and IAA. This tissue by itself will not normally produce such structures and it is evident that the period of growth of the cultures and the conditions under which this takes place are both important in influencing the final form of the explant and its associated structures (Skoog 1957).

8.1 Auxins.

The most consistent response of plant tissues to high concentrations of auxins is cellular proliferation (Thomson 1945).

However, different species of plants may respond to the same auxin in different ways, while different auxins applied to the same species may also produce different reactions. Blum (1941) has shown that although a group of similar plants treated with the same auxin has the same general reaction, individual plants, taken separately, may show reactions similar to those induced by other auxins. This emphasizes the inherent variability of plant material and conditions of the experiment and shows that the exact way in which a plant tissue will react to an applied auxin cannot therefore be predicted with certainty.

Because the literature concerning the effects of auxins on plants covers a very wide range of plants, this review will be restricted where possible to the reactions of sunflower tissues to these agents.

One of the earliest reports of tissue stimulation in sunflower by auxin was made by Snow (1935) who added ether extract of auxin from urine to a decapitated hypocotyl over the cut surface and to a point 8 mm below this and showed that the cambial zone within and between vascular bundles was stimulated and that further secondary xylem was formed in the bundles. This effect was evident 2 to 3 mm below the zone of application. In later experiments using a higher light intensity Snow (1935) showed that the cambium was stimulated 6 to 8 mm below the zone of application. Numerous cell divisions were shown to have occurred in the inner cortex while root primordia formed and pushed through the cortex into the gelatin containing auxin. In further experiments using 2 ppm of auxin "a" applied to the decapitated epicotyl the effects of the auxin were evident 20 to 30 mm below the zone of application.

Blum (1941) used sunflower plants six to seven weeks old to study the different responses of various auxins. The plants were decapitated above the third internode and the cut surface was treated with a 2% concentration of the auxin applied in lanolin. Indole acetic acid, indole butyric acid, indole propionic acid, naphthalene acetic acid and phenylacetic acid were used. Most of the responses were confined to the callus and the upper 3 to 4 mm of the decapitated stump. When no auxin was present in the lanolin applied to the cut surface no appreciable callus was formed, and secondary thickening and cell maturation in the original tissues were suspended. With auxin application all of the parenchymatous tissues near the cut surface began to proliferate. With most auxins the pith responded for some distance below the cut surface by some cell division and the lignification of other cells to form wound tracheids. Cell division and enlargement occurred in the cortex near the cut surface while the cambium contributed cells to the callus. Secondary thickening was uniform in all the plants treated with the auxins except those treated with phenyl acetic acid. Phloem parenchyma contributed to the organization of adventitious root tips through its derivatives in the callus, and also added to the callus formation. Root formation was most marked with the IAA treatment when many root tips appeared to be formed from meristematic cortical tissue. The endodermis was responsive to the application of most auxins for 1 to 2 mm below the cut surface, the Casparian strips remaining with the innermost cells. Within the callus, in the same plane and above the cut surface, a horizontal cambium developed from parenchymatous

cells of the xylem and pith: this was continuous with the vascular cambium. At the inner face of this horizontal cambium cells were cut off which developed into xylem - like elements which could become lignified.

Tissue development of the red kidney bean after inoculation with IAA is very similar to that described for the sunflower (Kraus, Brown and Hamner 1936). In the bean the endodermis was one of the most reactive tissues, cell divisions being induced up to 5 cm below the cut surface. In this plant callusing induced by IAA was typified by the formation of root primordia just below the zone of application. In some of the specimens examined the primordia were described as arising in the outer layers of the proliferating endodermis with the differentiation of vascular structures through the intervening tissues back to the xylem of the original bundles. In most cases, however, root primordia were described as arising from the division of ray cells between two adjacent masses of phloem; the phloem could also have contributed to their formation.

Kraus (1941) reported the occurrence of extra-vascular bundles formed mainly from the endodermis in the red kidney bean, on the application of l-tryptophane. This unusual bundle formation was not induced by the application of any other growth substances in the red kidney bean.

It has been reported by Reinders - Gouwentak (1965) that in non-dormant shoots cambial activity is stimulated throughout the shoots on the application of IAA. But in the work of Snow, Kraus et al and Blum, activity of the cambium was generally confined

to the top 3 or 4 mm of the stem below the point of application of the auxin. The limited reaction in Snow's experiments was noted by Reinders - Gouwentak, and was thought to have been due to the age of the plant tissues used. In incompletely differentiated tissues the cambium will not react to growth substances by radial growth until extension growth is complete, and the hypocotyls and epicotyls of Snow's experiments were used when the cotyledons had expanded to 2 cm in length, or the first pair of leaves to 5 or 6 cm. This explanation fails to account for the localized stimulus of the cambium in sunflower plants which were six to seven weeks old (Blum 1941).

Few studies have been made on the response of segments of plant stems to auxins. Beal (1940) used segments of the second internode of red kidney bean plants. After treating decapitated stems with 0.5% IAA in lanolin paste, pieces 2 to 15 mm long were cut from the shoots and placed, basal end down, in petri dishes containing solid White's medium. Segments 2 to 5 mm long showed slightly more swelling at the basal end at first, but were thickened throughout their length; later the apical end enlarged to a greater size than the basal end. The first tissues to show a response to the IAA were the cortex and the endodermis, followed by proliferation of the cambium, phloem parenchyma, and ray cells, as occurred in the bean described by Kraus et al (1936). Seventy-two hours after the application of IAA, root primordia began to develop at both apical and basal ends within 1 or 2 mm of the cut surface. In longer segments both ends formed callus and root

primordia, but the region in between, apart from elongating markedly, did not show any noticeable cellular change. The controls elongated as in the treated segments but no cellular changes occurred in the tissues. Because the segments continued to elongate in the medium, it is not surprising that no cellular changes were found in the middle of the treated segments since, as was noted previously, extension growth must be complete before radial growth can occur (Reinders - Gouwentak 1965).

Beal also made transections 0.5 mm or less in thickness from the apical swellings of stems 24 hours or more after they had been decapitated and treated with IAA. These sections were grown on solid White's medium where the tissues outside the xylem cylinder produced a large amount of proliferation.

No histological study has been described for 1 mm disks of sunflower hypocotyl grown on auxin-containing media as in the present experimental work. De Ropp (1947a) however, used tissues of the first internode of the sunflower in the form of 3 mm-long segments, which were grown in the dark on media containing different concentrations of IAA, IBA or NAA. At concentrations of 0.01 ppm IAA or IBA abundant root formation resulted. Up to seven roots of approximately equal length, evenly distributed around the stem, were shown to arise from the interfascicular tissue. With IBA secondary roots grew out in an even series well behind the main growing point while with IAA they grew more irregularly but more vigorously, often challenging the dominance of the primary root. In the pieces of tissue treated with NAA fewer roots emerged and usually only one of these developed to any size, sending out an

unevenly spaced series of laterals.

An auxin concentration of 10 ppm produced proliferation of the cambial layer which gave rise to loosely connected translucent material growing out into loose elongated elements resembling root hairs. At concentrations of 0.1 ppm IAA these stem fragments showed, according to de Ropp, the development of xylem-like tissue between the vascular bundles. At this concentration root growth was not completely suppressed, but the roots formed often showed swelling and partial disintegration of the cortex.

Apart from the gross observations made, no study of the cambium or endodermal tissues was carried out, nor was the exact origin of root primordia described. For root primordia to be initiated a particular tissue must have been stimulated, and the degree of stimulus apparently varied depending on the auxin used.

In this study de Ropp also observed the influence of growth substances on pieces of bacteria-free sunflower crown gall tissue. His only conclusion from this was that IAA did not alter the tissue structure at all, but that growth was inhibited at high concentrations of IAA. Struckmeyer, Hildebrandt and Riker (1949) made a detailed study of bacteria-free crown gall tissue of the sunflower. Pieces of tissue were grown for six weeks on media containing high and low concentrations of IAA, NAA, or parachlorophenoxy acetic acid.

Except for differences in wet weight no macroscopic effects were observed; this agrees with de Ropps' findings. On microscopic examination, however, it was found that high concentrations

of IAA and IBA (100,000 ppm and 1,000 ppm respectively) considerably inhibited meristematic activity. Both hypertrophic and hyperplastic cells were larger than those of the tissues grown without auxin or with a lower concentration of the growth substance. The tracheal elements were fewer in number at high concentrations than in the controls. At low concentrations (0.00001 ppm, 0.001 ppm or 0.1 ppm) the hypertrophied cells were also larger than in the controls and there were more tracheal elements. The greatest differences were found in a comparison between tissues grown on high and low concentrations of growth substances.

The work of de Ropp (1947a) and of Struckmeyer et al (1949) using different concentrations of auxins leads to the realization that even a single tissue type may react in varying ways to auxins, depending on the concentration used. The reactions of tissue cultures freed from the restraint of the whole plant are different from changes occurring within the plants. Gautheret (1959) has summarized the reactions of tissue cultures to various concentrations of auxins. He classifies sunflower tissue as being stimulated to produce roots and to become hyperhydric on the addition of auxin. Hyperhydric tissues are not able to be cultured over long periods.

The formation of roots in tissue cultures has also been reviewed by Gautheret who concludes that their origin and mode of organization may be very variable. In some tissues, for example carrot, roots are formed from the xylem, phloem, or cambium, and it has been shown that the tissues of origin may vary with the concentration of auxins applied. Once a primordium has been initiated and cells

become organized into a meristem, further growth of the root and penetration through the tissues may be inhibited because of high concentrations of auxin used. In cultures of tobacco stems Sterling (1956) described the origin of roots as being from the cambium, and de Ropp (1947a) also found roots formed from cambium tissue of Vinca rosea. Gautheret (1959) concludes that the origin of roots in tissue culture has been examined frequently with varying descriptions but it is likely that they are formed from the cambium present in the tissue pieces.

The origin of roots in decapitated stems or in stem segments under the influence of auxins appears to be mainly from the xylem ray cells with some contributing phloem cells, or from proliferating endodermal cells, but in tissue cultures roots seem to arise from quite a different tissue, namely the cambium.

8.2 Bacterial Agents.

a) Agrobacterium tumefaciens. It appears to be widely accepted that with crown gall infection, a growth pattern occurs similar to that induced by synthetic auxins in relatively high concentrations (Thomson 1945). However, few studies directly comparing the effects on plants of A. tumefaciens and of auxins have been made. Braun and Stonier (1958) in their review of the morphology and physiology of plant tumours state that IAA-induced overgrowths bear a close resemblance to crown gall tumours both morphologically and histologically.

Kraus, Brown and Hamner (1936), in studying the effects of

IAA on the red kidney bean, directly compared the proliferation of endodermal cells within 3 cm of the apical callus with tumour strands of crown gall, and stated that the histological responses of the cells to IAA were almost exact duplicates of those described for crown gall by Smith, Brown and McCullock (1912). They themselves did not grow the two treatments under comparable conditions. Link, Wilcox and Link (1937) have said that by applying IAA in different concentrations to bean and tomato plants, the whole range of callusing including tumour formation, can be produced. Their only histological study, however, was carried out on beans after the application of an extract from A. tumefaciens cultures, so that their statement was not supported by experimental data.

The frequent analogies between the effects of A. tumefaciens and growth substances on plants may have arisen from the fact that it is the host rather than the inciting organism which determines the nature of the response in many cases (Levine 1940). It has been emphasized by Kupila (1958) that the character of secondary growth and the degree of polyploidy of normal stem cells are all factors which influence a growing gall. The processes of normal wound regeneration in their turn affect the development of crown gall tissue. These factors combine to produce the differences in crown gall development noted in different species. There are few cases in which the effects of A. tumefaciens-inoculation and of the application of IAA have been directly studied in a single species under the same conditions. De Ropp (1947a) compared the growth of segments from the first internode of sunflower with that of pieces of bacteria-

free tumour tissue, on media containing growth substances. A description of the stem segments was made in the previous section; the pieces of bacteria-free gall tissue showed no evident change except for a slight inhibition of growth. The two tissues described are not, however, comparable in character and origin.

Appler (1951) compared the structure of decapitated seedlings of Helianthus annuus inoculated with A. tumefaciens and with IAA. The auxin calluses could not be separated, externally, from the bacterial tumours. On histological examination, the cell and the nuclear size of callus cells induced by auxin were smaller than in crown galls but the same nests of tracheids occurred in both cases, unconnected with the vascular bundles. In the anatomical structure, then, no difference could be found between IAA induced galls and crown galls formed under the same conditions in sunflower.

De Ropp (1950) has stated that "it is generally recognized that the action of A. tumefaciens and of IAA differ from one another in several respects". He has made one of the few reports of authentic differences between crown gall and IAA-induced overgrowths, using pieces of carrot root inoculated in the central region. Overgrowths induced by A. tumefaciens consisted of a disorganized cambium enclosing xylem tracheids and parenchyma. On the outside some phloem tissues lacking sieve tubes were formed and islands of rapidly multiplying cells were also formed in this area. These formations combined to give a spherical pattern of growth. In contrast to this, tissues treated with 3% IAA in lanolin produced almost entirely root primordia. When 10 ppm of IAA was added to

the medium a more generalized proliferation occurred and enlarged hyperhydric cells were formed. The structure of the cambium was lost as the result of this proliferation.

White and Braun (1942) examined the habit of tissues from sunflower which had been induced to proliferate before removal from the plant, by the action of indoleoxaloacetic acid or A.tumefaciens. Tissues from the chemically induced growths were externally compact and green, and sometimes formed roots. Well-organized vascular elements were often differentiated giving the explants a fibrous or woody character. Tissue cultures of crown gall origin (from secondary tumours which were apparently sterile) grew much more rapidly than the chemically-induced overgrowths, producing a spongy, open friable type of tissue in which scattered vascular elements were formed. These vascular elements were not grouped together to form woody areas in the cultures. In no cultures from secondary crown galls were roots ever observed to form.

The detailed structure of crown gall has been studied extensively by Kupila (1958, 1963). The development of crown gall in sunflower is essentially different from that in pea and tomato, the anatomy of sunflower galls being more orderly than in the latter two plants. In the sunflower, proliferation in the pith and cortex produces cells similar to the original tissue so that it is difficult to differentiate between normal and tumour tissue. Derivatives from the tumour cambium form tracheids on the pith side and phloem elements towards the outer surface, showing that the tissues differentiate in a similar manner to normal parts of the

stem. Rapid proliferation of some parts of the tumour cambium may cause protruberances. In the pea and tomato, tumours are characterized by disorganized cells of various sizes arising from many separate growth centres, these latter becoming linked between the woody elements which have developed.

Kupila (1963) has noted that her description of sunflower crown galls differs from that of Braun and Stonier's (1958) and ascribes this to the difference in virulence of the bacteria used, indicating that this property of the bacteria may be of more importance in the character of induced tumours than has so far been supposed.

b) Other Bacterial Agents. Bacillus megaterium was found to stimulate the proliferation of vine tissues (Fallot 1958, 1960, 1964). This bacterium also produced very extensive proliferation on explants of dormant Jerusalem artichoke tubers. Besides the production of more normal vascular bundles, the newly-formed tissues contained aberrant bundles with very little lignified xylem. A number of isolated tracheids also occurred scattered throughout the tissues, while a cork cambium formed on the outside of the explant. The formation of callus in the presence of B. megaterium was less generalized than that resulting from the application of 1 ppm of IAA or NAA. Fallot stated that there were no known substances which could produce such a structure in artichoke explants.

Fallot (1964) further tested the ability of Azotobacter chroococcum, Beijerinckia indica and Sarcina lutea to cause proliferation on tissues of artichoke. All of these bacteria stimulated proliferation but only in the case of A. chroococcum was a

histological study of the tissues made. The structure of the callus was entirely different from that resulting from the action of B. megaterium. In this case the newly-formed tissue contained independent vascular bundles arranged in a single layer, with phloem towards the outside and xylem towards the inner mass of tissue. The arrangement of tissues was similar to that resulting from the action of IAA.

8.3 Virus Wound Tumour.

The wound tumour virus may cause a variety of symptoms in a number of different species of plants. These symptoms include leaf curling and distortion, small tumours on veins, leafy outgrowths from the underside of veins, and some stem tumours, but the main effects of the virus are irregular vein enlargement and tumours on the roots. The only plants known to produce stem tumours are Melilotus alba and M. officinalis.

Root tumours have been studied in detail by Kelly and Black (1949) and by Lee (1955). These investigators have shown that in the root 85% of the tumours are formed at the base of emerging lateral roots. The meristematic cells initiating the tumour are formed in the pericycle opposite the primary phloem but not in direct contact with the endodermis. The tumour develops by cell division rather than by cell enlargement. Xylem cells are formed at the tip while at the base of the tumour a primitive type of phloem develops in which sieve tubes are absent; meristematic tumour cells separate these two tissues. As in the phloem,

primitive xylem cells are formed which are short and possess end walls, and are reticulately thickened. Extensions of the xylem reach into the meristematic region. Subsequent growth of the root tumours does not follow any particular pattern although the xylem may show connections with the vascular tissue of the root. A comparison of the tissues of the tumour with those of the root shows that although many of the same tissues occur in both, tumour tissues are very distorted and the xylem and phloem cells are less differentiated than in normal tissues.

The sweet clover stem tumours have a different origin from root tumours. They arise in the phloem either from primary phloem fibres, phloem parenchyma, or occasionally from the phloem procambium. The virus is transported through the plant in the phloem so that it is understandable that it should affect the tissues nearest to it. Stem tumours may develop in much the same way as root tumours with xylem towards the outside; but more often, xylem is differentiated in the inner part of the tumour and connected to the vascular strands of the stem. The xylem is covered by several layers of meristematic tissue and phloem develops to the outside of this.

In the wound tumour early growth is more organized than with other neoplastic growth; xylem and phloem cells in orderly array are separated by a meristematic region. This growth may be compared with that of sunflower crown galls although a greater degree of disruption occurs with the aging of the latter.

8.4 Genetic Agents.

a) Nicotiana hybrid tumours. In the most recent review of the genetic tumours produced in hybrid crosses of Nicotiana glauca and N. langsdorffii Kehr(1965) dismisses the histology of these growths as being similar to that of wound callus and crown gall; but wound callus and crown gall may show quite different histological characters. This description does not agree with that made earlier by Kehr and Smith (1954) in which Levine's (1937) conclusions were followed: namely that genetic tumours differ histologically from crown gall tumours, the former being more organized than the latter with root and shoot initials.

Genetic tumours were first described by Kostoff (1930) who said that their structure could range from fasciations and witches' brooms to tumours whose composition was similar to that of crown gall, consisting mainly of parenchymatous tissue with vascular bundles composed of short disconnected cells irregularly arranged. Whitaker (1934) described the tumours as being externally similar to crown gall, but histological investigation showed them to consist mainly of parenchymatous tissue associated with scattered vascular elements. Near the surface of the tumour small meristematic areas were noted.

A later report by Levine (1937) compared crown gall on both N. langsdorffii and N. glauca with the genetic tumours formed on the hybrid of these two. This was the first direct comparison of the two types of neoplastic growth. N. langsdorffii produced only small crown galls which, after three to four months, became hard and covered with cork. N. glauca in contrast was readily induced to

form galls and the general structure of these was typical of crown gall in other species of tobacco. The genetic tumours on the hybrid were found to have soft callus tissue at the base of the overgrowth, continuous with the pith of the stem. Surrounding this callus tissue, small areas of deeply staining embryonic tissue gave rise to closely compressed leaf and bud elements which were connected with the host stem by vascular bundles. Nests of irregular xylem tracheids or vessels, common in crown gall, were not formed. In the spontaneous tumours of the tobacco hybrid no fundamental irregularities occurred in the arrangement of the plant tissues. Root tumours, although macroscopically like crown gall, were found to have an organized structure similar to that of the stem tumours.

Brieger and Forster (1942) studied the development of genetic tumours. They found that cells adjacent to necrotic tissue were initially activated in the primary cortex between the epidermis and collenchyma. Later the whole region became activated and the cortex layers degenerated. Although large tumours were formed at this stage they were not connected with the xylem. Finally, however, the proliferation extended into the pith, forcing open the vascular cylinder, and connections were established with the vascular tissue. Brieger and Forster showed that genetic tumours were initiated in mature cells of the cortex and no meristematic areas were involved in their early stages of growth. No compressed leaf and bud elements, as described by Levine (1937) were observed, but it is likely that these did not develop until a later stage of growth in the tumours.

These genetic tumours formed in hybrids between N. langsdorffii and N. glauca are similar to crown gall in that their initiation is in areas adjacent to necrotic tissue, where the cells are mature.

The technique of tissue culture from tobacco hybrid tumours has been used to study further properties of this material. White (1944) grafted pieces of tissue from a genetic tumour to N. glauca and compared their growth with grafts of normal cells. The genetic tumour tissue used for the grafts lost its organoid character when grown on an agar substratum although buds could be produced by altering the conditions of culture. With the genetic tumour tissue grafts, the galls formed were small in comparison with those formed by grafting bacteria-free crown gall tissues on Helianthus, but they were essentially similar in general appearance. Histological examination showed that the two types of tissues were similar in structure with characteristic disoriented masses of cells occurring at the boundary between the tumour and the healthy cortex, and between tumour and host xylem. The rapidly-growing outer part of the tumour was even more disorganized with scalariform cells scattered through the tissue.

It would appear that, like virus wound tumours, genetic tumours of Nicotiana hybrids are essentially different from crown galls when induced on whole plants. Growth is more organized than with crown gall. However, under conditions of tissue culture and grafting the genetic tumour tissues do resemble those of crown gall. It may be that once alteration has occurred in the tissues under the influence of the tumour-inciting agent, normal restraints are no

longer imposed on the cells, and under tissue culture conditions similar cell types are formed.

b) Spruce Tumour. A further plant growth which may have a genetic origin is White's spruce gall. This gall has not been studied in its early stages since sections can be made only when the gall becomes macroscopically obvious on the branches of Picea glauca.

On sectioning, White (1958) found that the galls must arise in the bud and not from secondary cambium, since they always extend right into the pith. Galls of variable sizes do occur but all are initiated by a single cell and their final size is dependent on the number of lateral divisions which occur after their initiation. A small gall may be shown to have existed as a single file of cells for up to twenty years before lateral divisions occurred.

When the spruce gall was first described histologically (White and Millington 1954) the cells of the gall were said to have walls the same thickness as ordinary cells, but the lumens of the cells were larger and less regular. Later White (1958) described the gall sectors as being macroscopically recognizable because of thinner cell walls, as a result of which the cell lumens were slightly larger. The difference in cell size is presumably important in detecting single strands of gall cells. In larger galls, however, the main difference between gall cells and normal cells is in the prolonged cambial division of the former. This activity increases the amount of xylem tissue produced, although the bark on the outside of the tumour is similar to that of normal bark, showing that little or

no extra phloem tissue is formed. Galls produced on the spruce may appear very large but their internal structure shows that unlike the types of abnormal proliferation studied previously, the only modification produced is the increased xylem formation; the original tissues are not invaded. Whereas in other galls mainly parenchymatous tissue was produced with some meristematic growth centres and the differentiation of vascular elements, the spruce gall merely shows an increased production of xylem tissues due to an activated section of cambium. Since no causal agent has been found, it seems likely that a somatic change may have occurred in a section of the cambium in the bud giving rise to the gall sectors.

All the types of abnormal growth studied are different from one another and it would appear that each agent induces a different type of tissue response. The main way in which this can be verified is by testing the different agents on the same plant species, and noting the differences and similarities in the resulting tissues. All too often this aspect of research has been neglected and comparisons have been made between tissues not strictly comparable.

CHAPTER NINE

HISTOLOGY OF INOCULATED HYPOCOTYL DISKS

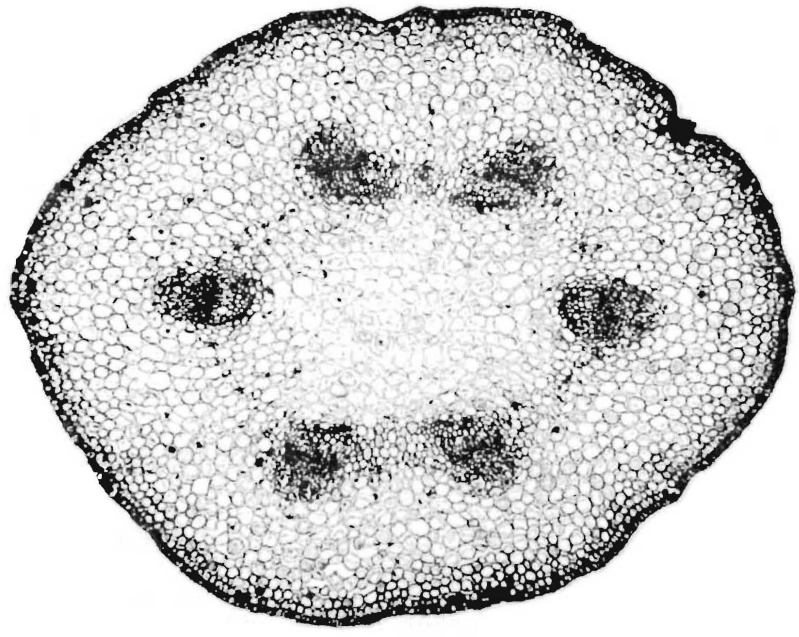
The general morphology of sunflower disks treated with either Escherichia coli, Agrobacterium tumefaciens or sterile medium differed markedly after growth on de Ropp's medium. Further differences appeared when the inoculated disks were grown on media containing different concentrations of indole acetic acid. From the study of fresh and dry weights of the disks it was determined that all disks did expand, with an increase in weight, but for any given treatment it could not be predicted with certainty the way in which the disks would react. Generally the disks from the top of the hypocotyl increased more in length, while those taken from lower down grew more in diameter.

An investigation of the histological structure of sunflower hypocotyl disks was carried out to determine how the different tissues reacted to the presence or the absence of the bacteria, bearing in mind the inherent variability of the material.

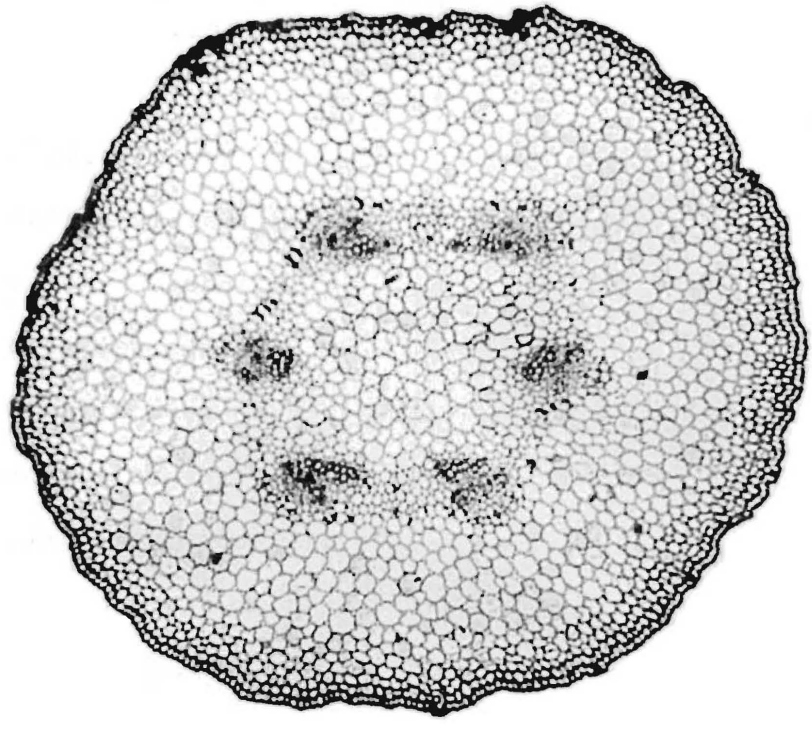
9.1 Preparation of Material for Sectioning.

All hypocotyl disks were fixed in a solution of formalin acetic alcohol comprising 5% formalin, 5% glacial acetic acid and 45% ethyl alcohol. The tissues were then dehydrated with tertiary butyl alcohol and embedded in paraffin wax m.p. 63°C (Johansen 1940). The disks were cut either on a Cambridge rocker microtome giving sections 10μ thick, or on an M.S.E. rotary microtome giving 12μ thick sections. The sections were then stained with safranin and

198 a.



a) T.S. of a light-grown hypocotyl at time of inoculation - 5 days



b) T.S. of a dark-grown hypocotyl at time of inoculation - 4 days

FIGURE 9.1 STRUCTURE OF LIGHT AND DARK-GROWN HYPOCOTYLS AT THE TIME OF INOCULATION (Mag. x 50)

fast green and mounted in a synthetic neutral mountant, "de Pex". Using these stains, phloem tissue could not readily be distinguished from the surrounding parenchyma, whereas lignified and suberised cells were clearly stained.

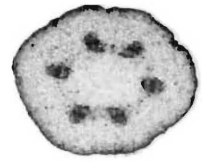
Histology of Disks Grown in the Light.

Five days after germination at the time of preparation of the disks, the hypocotyl consisted of normal stem tissues (Figure 9.1a and 9.2a). The epidermis was composed of small isodiametric cells and the cortical parenchyma showed a gradation from small cells near the epidermis to larger cells in the inner cortex. Up to five secretory canals were situated outside each vascular bundle in the inner cortex, while the endodermis, which was clearly marked by a concentration of starch grains, lay immediately inside the secretory canals, encircling the vascular cylinder. The vascular tissue in the part of the hypocotyl used for disks was composed of six discrete vascular bundles. Four of these were arranged in two pairs and were continuous with the midribs of the cotyledons, while the other two lay between these pairs and became intercotyledonary bundles (Thiel 1934).

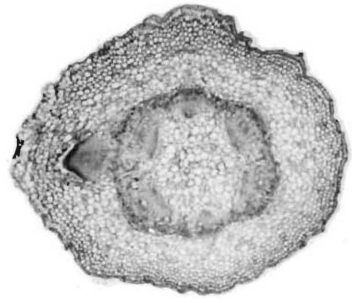
The primary xylem vessels were annularly or spirally thickened and were separated from the cambium by xylem parenchyma. Small areas of phloem were present on the outside of the cambium of each bundle.

9.2 Uninfected Disks.

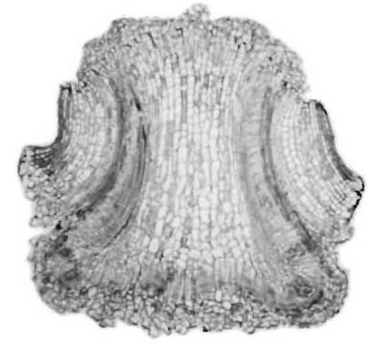
a) Medium without added IAA. After two weeks of growth, disks



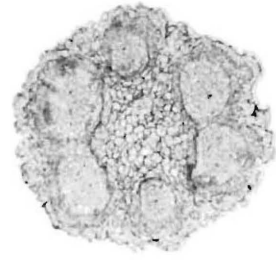
a) T.S. at time of inoculation - five days



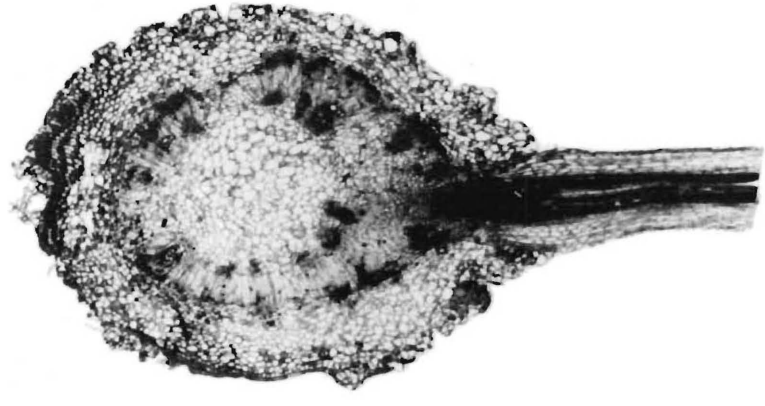
b) T.S. two weeks after inoculation



c) L.S. two weeks after inoculation



d) T.S. of wound cambium at lower surface two weeks after inoculation

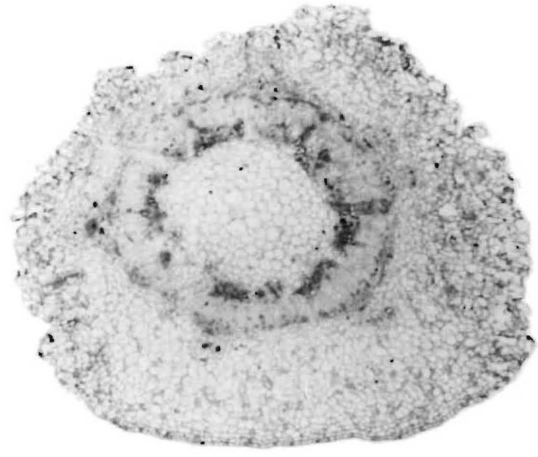


e) T.S. five weeks after inoculation

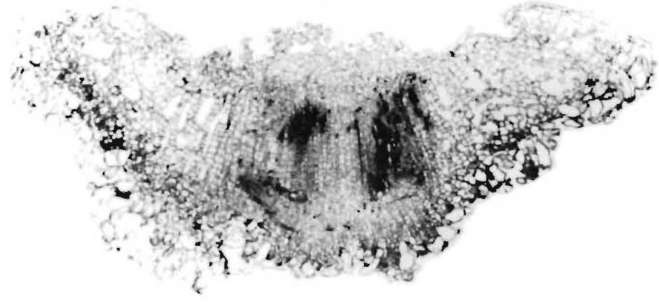
FIGURE 9.2 UNINFECTED DISKS GROWN IN THE LIGHT ON MEDIUM WITHOUT ADDED IAA (Mag. x 12.5)

inoculated with sterile synthetic medium retained their epidermis while the cortical tissues expanded to about three times their original size. The vascular bundles were joined by an interfascicular cambium which gave rise to xylem parenchyma with an occasional xylem vessel at its inner face. Further towards the base of the disk the xylem of the vascular bundles continued to develop with the formation of a majority of small vessels interspersed with larger vessels. Secondary xylem formation was concentrated near the protoxylem, helping to maintain the original vascular pattern. Like the cortex, the pith parenchyma expanded, although not to quite the same extent. Near the base of one disk examined, a root primordium arose from between the vascular bundles, in the interfascicular cambium or parenchyma between this cambium and the endodermis (Figure 9.2b). The origin of roots throughout this work appeared to be from this region in the original tissue.

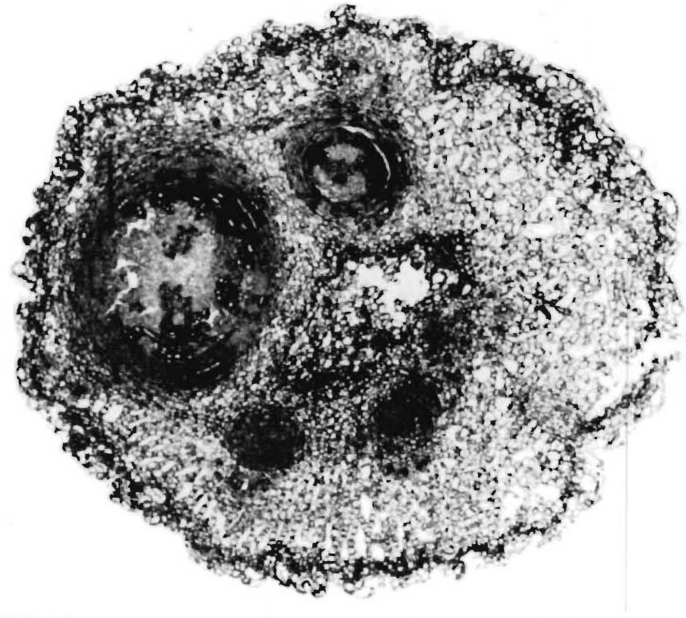
Growth of the original tissues was readily observed in transverse section but from a longitudinal section it could be seen that wound cambia were present, parallel to both upper and lower cut surfaces (Figure 9.2c). These cambia were formed from the vascular cambium, xylem and phloem parenchyma, and incorporated some pith tissue. The origin of the wound cambium was similar to that described by Blum (1941). At the upper surface, the cambium cut off mainly parenchyma tissue and was not as well developed as at the lower surface. There, the original vascular tissue was extended and the cambium formed below this, encircling the ends of



a) T.S. three weeks after inoculation



b) L.S. three weeks after inoculation



c) T.S. five weeks after inoculation - wound cambium

FIGURE 9.3 UNINFECTED DISKS GROWN IN THE LIGHT ON MEDIUM CONTAINING 0.01 PPM IAA (Mag x 12.5)

the vascular bundles (Figure 9.2d) as well as continuing across the pith (Figure 9.2c). Occasional whorls of reticulately lignified tracheids lacking any particular orientation developed from the inner face of the cambium, while the majority of cells at the outer face were parenchymatous with an occasional phloem element.

After three weeks of growth, additional lignification of the existing xylem elements occurred although there was little further expansion of the ground parenchyma. More activity of the wound cambia contributed to the increasing amount of parenchyma at both surfaces. One or two root primordia had developed near the base of the disk and these continued to grow, penetrating the cortex.

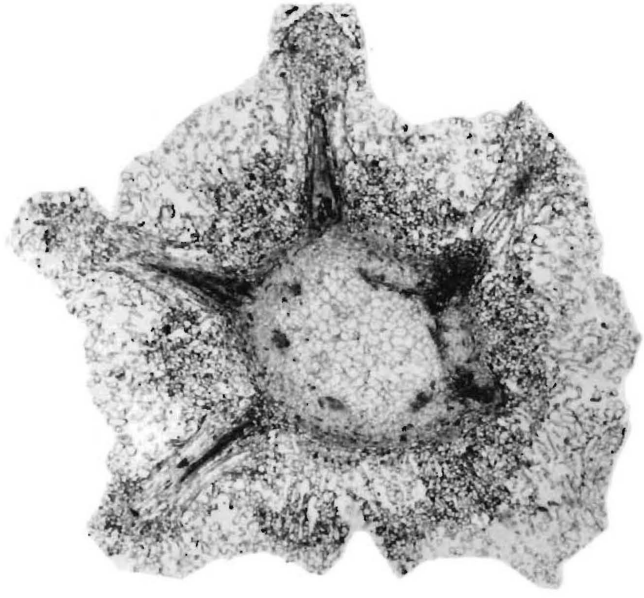
Little further change occurred in this basic structure after five weeks' growth although there was increased growth of the roots (Figure 9.2e).

b) Medium + 0.01 ppm IAA. In general, hypocotyl disks grown in the light on a medium containing 0.01 ppm IAA showed a gross morphological response similar to that of disks inoculated with E. coli and grown on a medium without added auxin (see Chapter 5) but histologically these two responses were different. After three weeks of growth the cells of the parenchymatous tissues had mostly expanded, while some pockets of cells in the cortex and pith were stimulated to divide (Figure 9.3a). These stimulated cells were often mature since after division the outline of the original cell wall remained (Palser 1942). A continuous vascular cambium was present which gave rise mainly to xylem parenchyma. Secondary xylem vessels were concentrated near the protoxylem of

each bundle. The quantity of vascular tissue produced was similar to that in uninfected disks grown without auxin. A few phloem fibres were formed outside each of the vascular bundles. In longitudinal section (Figure 9.3b) it could be seen that the cortical cells expanded and lacunae were formed between the filaments. The wound cambia formed at both upper and lower surfaces were more extensive than in previous treatments since the cortical tissue, in addition to vascular parenchyma, appeared to contribute a little to the total proliferation. In this particular example cell division occurred throughout the pith and the wound cambium at the upper surface seemed to be a continuation of this, rather than a more organized structure. At the lower surface too, the wound cambium was not as organized as in disks grown without auxin. The cells directly in contact with the medium expanded but the collapse shown by parenchyma cells in disks inoculated with E. coli (Figure 9.5b, c) was not evident.

After five weeks' growth the most noticeable change in the disks was a marked increase in the lignification of the vascular tissues. Vessels formed an almost continuous band between the original vascular bundles, while the number of phloem fibres capping the vascular bundles increased. Lignified wound tracheids inside both wound cambia also added to the total increase in thickening (Figure 9.3c). Usually two or three long roots were formed in disks grown on this medium.

c) Medium + 0.1 ppm IAA. Disks grown on a medium containing 0.1 ppm IAA reacted by producing a large expansion of the cortex.



T.S. three weeks after inoculation

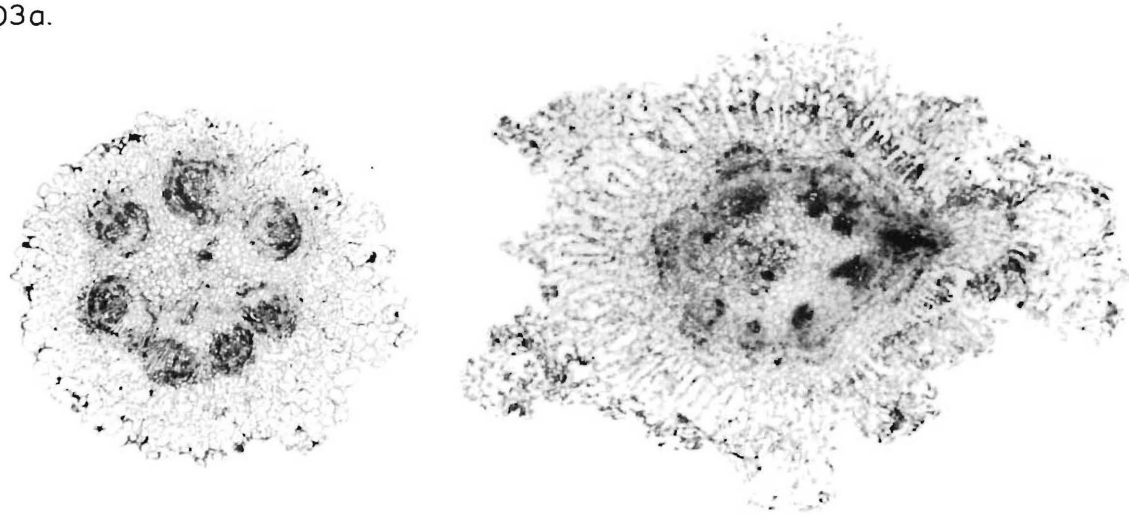
FIGURE 9.4 UNINFECTED DISKS GROWN IN THE LIGHT ON MEDIUM CONTAINING 0.1 PPM IAA (Mag. x 12.5)

This mass of tissue was added to by the activity of the lower wound cambium. The pith parenchyma was also stimulated to expand and occasional cell divisions were also found. In disks three weeks old little secondary thickening of the vascular bundles had occurred although a wide interfascicular cambium had been formed. This concentration of IAA in the medium was sufficient to inhibit the lignification of the vascular tissues even after five weeks of growth. The interfascicular region was stimulated by the auxin to form five large roots. The cortical tissue of these roots acted in the same way as the cortex of the disk, forming filaments of cells separated by lacunae (Figure 9.4).

Tissues showed very little change after five weeks' growth. Expansion of the parenchyma was complete and no further development of the vascular tissues had occurred.

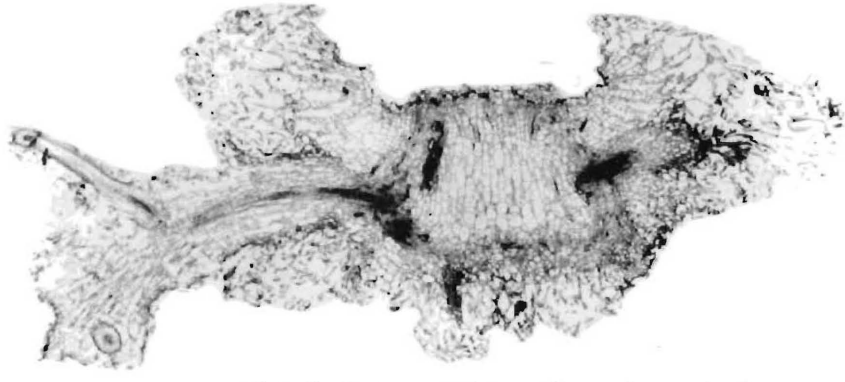
9.3 Disks Inoculated with E. coli.

a). Medium without added IAA. After eleven days' growth disks inoculated with a 24-hour E. coli culture showed that the epidermis, although still intact, had become stretched through expansion of the inner tissues. Most of this expansion had developed from the lower cortical parenchyma where the normal cell pattern, as in uninfected disks, was disrupted by large thin-walled cells some of which had collapsed. A well-defined cambium cutting off mainly parenchyma cells linked the vascular bundles. Lignification of the xylem within the bundles had occurred, the pattern of small regular vessels being disrupted by larger vessels. Above each bundle a group of

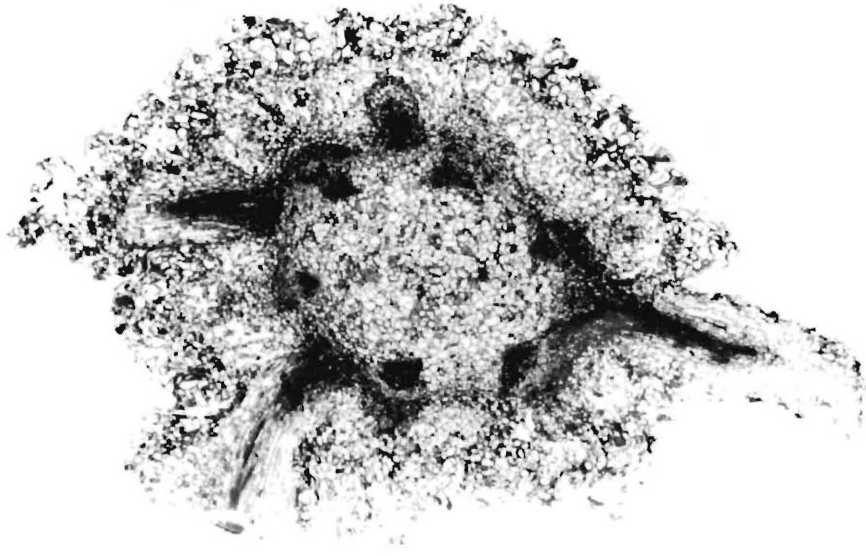


a) T.S. eleven days after inoculation - wound cambium

b) T.S. two weeks after inoculation



c) L.S. two weeks after inoculation



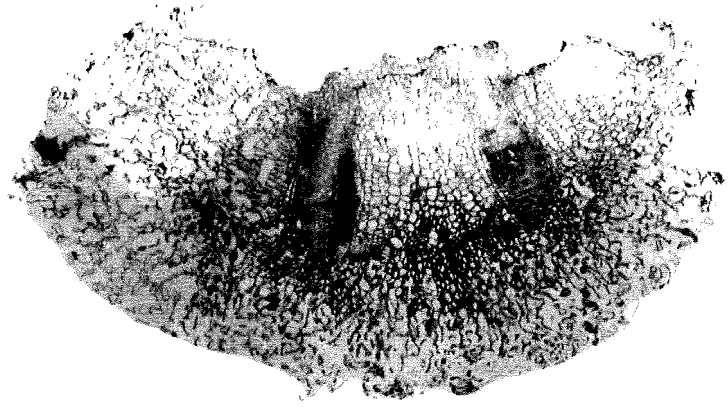
d) T.S. five weeks after inoculation

FIGURE 9.5 DISKS INOCULATED WITH E.COLI AND GROWN IN THE LIGHT ON MEDIUM WITHOUT ADDED IAA (Mag. x 12.5)

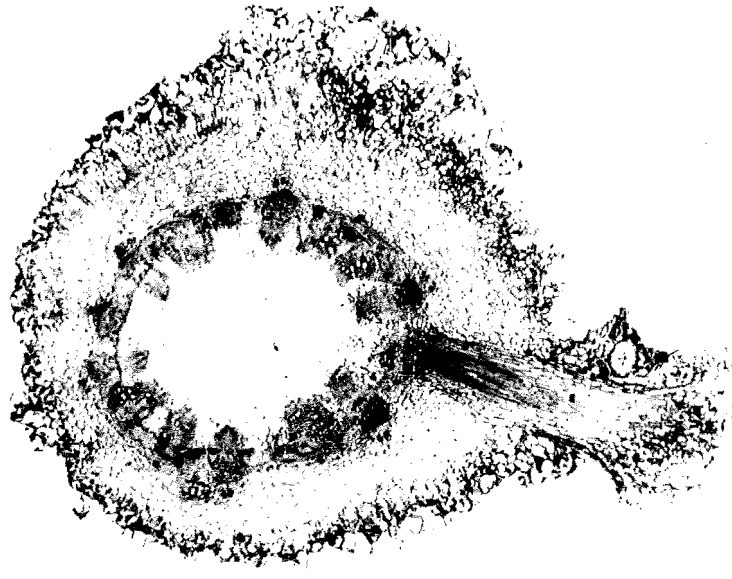
phloem fibres had differentiated. A root primordium emerged from between vascular bundles near the base of one disk, in the same relative position as described for the control. As in uninfected disks grown without IAA wound cambia, formed from the vascular cambium and associated tissues, crossed the pith parallel to both cut surfaces (Figure 9.5a). At the lower surface in particular, large irregular shaped tracheids with pitted or reticulately thickened walls traversed the pith and formed whorls around the base of the vascular bundles; these tracheids lacked any particular orientation.

After two weeks of growth a massive expansion of the cortex had taken place (Figure 9.5b). The epidermis of disks was pushed out at right angles to its original position by the cortical cells which had expanded in filaments separated by large lacunae (Figure 9.5c). Further lignification of the xylem had occurred, with a number of large vessels being formed near the cambium. The wound cambia continued to grow from the lower surface where the parenchymatous cells so formed expanded in a similar manner to the cells of the lower cortex. It was this tissue which was responsible for the external 'fluffy' appearance of the sides of disks treated with E. coli (Figure 5.3b). Large roots were formed from tissues in the original part of the disks; the cortex of these was also influenced by the bacteria, becoming filamentous like the cortex in the main body of the disks.

Expansion of the cortex was complete after five weeks' growth and lignification of the vascular tissues continued. Roots also



a) L.S. three weeks after inoculation



b)T.S. five weeks after inoculation

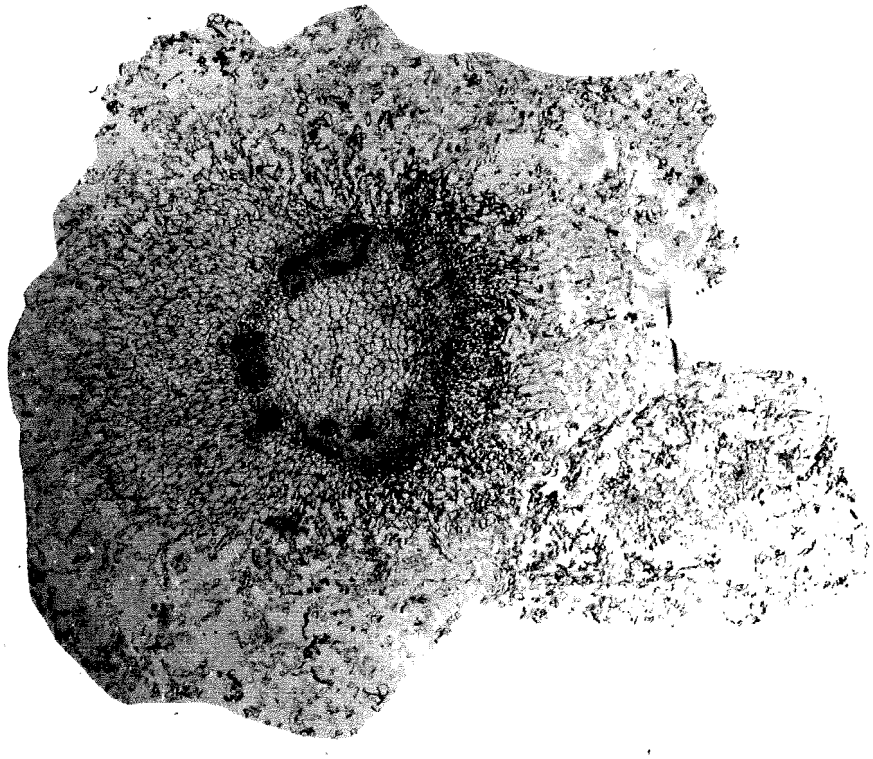
FIGURE 9.6 DISKS INOCULATED WITH E.COLI AND GROWN IN THE LIGHT ON MEDIUM CONTAINING 0.01 PPM IAA (Mag. x 12.5)

increased in size and up to six well-developed roots were formed in the disks depending on the seedling-light treatment (Figure 9.5d).

b) Medium + 0.01 ppm IAA. Disks inoculated with E. coli and grown on a medium containing 0.01 ppm IAA showed the influence of both the growth substance and the bacteria. In the majority of disks the parenchymatous tissues, particularly the cortex, expanded greatly, while large lacunae developed between the rows of cortical cells; cell divisions occurred in the pith. In disks three weeks old little secondary thickening occurred although a continuous interfascicular cambium had developed. The upper wound cambium was more highly organized than in disks without the bacteria (Figure 9.6a). This cambium was wide and gave rise to a large number of small, closely packed parenchyma cells. The lower wound cambium was also more organized than in the previous treatment, cutting off parenchyma to its outer face, and some whorls of tracheids to the inside. The parenchyma produced at the outer face of the cambium together with the cortical cells in the lower part of the disk showed the massive expansion and disintegration characteristic of E. coli-inoculated disks.

After five weeks of growth an increase in the secondary thickening of the xylem occurred. Large vessels extended throughout the xylem to the cambium, and phloem fibres capped the original vascular bundles (Figure 9.6b).

c) Medium + 0.1 ppm IAA. When grown for three weeks on this medium the cortex of disks inoculated with E. coli expanded to a



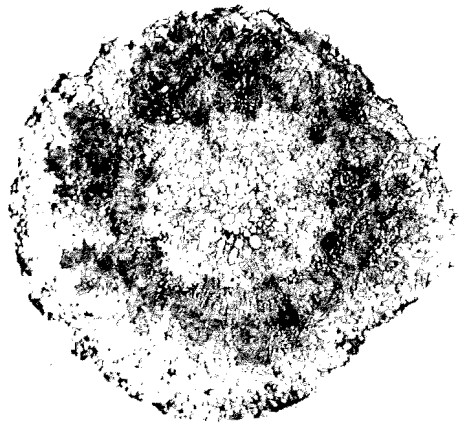
T.S. three weeks after inoculation

FIGURE 9.7 DISK INOCULATED WITH E.COLI AND GROWN IN THE LIGHT ON MEDIUM CONTAINING 0.1 PPM IAA (Mag. x 12.5)

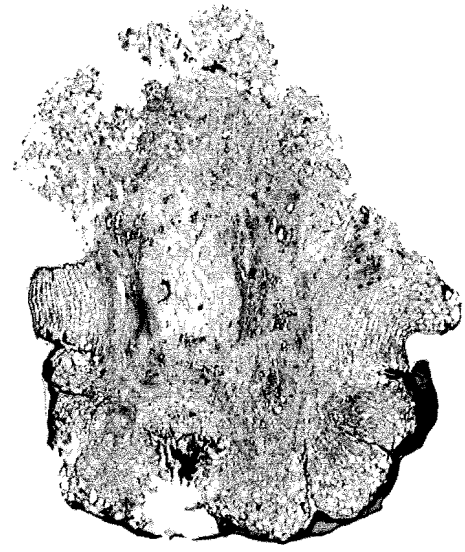
greater extent than that of uninfected disks. The epidermis was again displaced by the expanding inner tissues. Some cell division, as well as expansion, was evident in the pith parenchyma. Between the vascular bundles a small interfascicular cambium was sometimes formed. A little secondary thickening in the xylem occurred near the protoxylem and after five weeks some phloem fibres were present capping the vascular bundles (Figure 9.7)

9.4 Disks inoculated with A. tumefaciens.

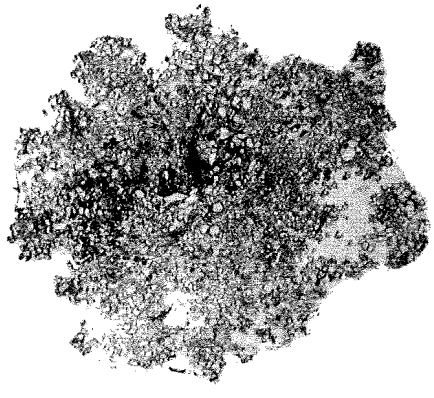
a) Medium without added IAA. In disks inoculated with A. tumefaciens the cortical parenchyma underwent some expansion but after eleven days' growth the cells of the outer cortex tended to disintegrate rather than divide (Figure 9.8a). Throughout most of the disk, but particularly near the upper surface where the bacteria were introduced, cells of the inner cortex divided and tracheids formed in whorls; these were frequently encircled by a disorganized cambium. This tissue formed almost a second vascular cylinder enclosing the original bundles. In the vascular bundles themselves, increased lignification occurred near the protoxylem. An interfascicular cambium joined some of the bundles, but this was disrupted in places by whorls of tracheids. A large amount of cell division was also induced in the pith and occasional tracheids were scattered throughout this tissue. The lower part of the disk was more like the uninfected controls in that the vascular bundles had become rounded off into whorls of parenchyma surrounded by tracheids. Between these whorls three root primordia arose in one disk examined.



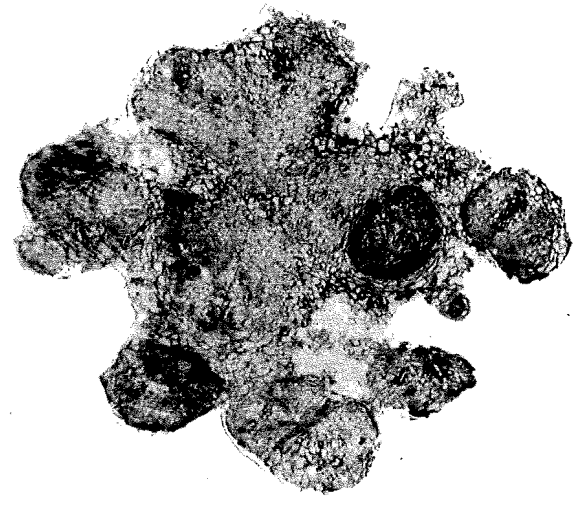
a) T.S. nine days after inoculation
Medium without added IAA



b) L.S. three weeks after inoculation
Medium + 0.01 ppm IAA



c) T.S. proliferation five weeks after inoculation



d) T.S. proliferation five weeks after inoculation
Medium + 0.1 ppm IAA

FIGURE 9.8 DISKS INOCULATED WITH A.TUMEFACIENS AND GROWN IN THE LIGHT ON MEDIA CONTAINING 0, 0.01 AND 0.1 PPM IAA (Mag. x 12.5)

After three weeks of growth the disks showed a continuation of the lignification of the upper surface tissues. In one of the disks examined, an even greater disruption of the tissues occurred such that nearer the base of the disk, mixed xylem and phloem elements were grouped into a very irregularly shaped ring. As in disks nine days old, tracheids formed throughout the pith.

A continuation of this general growth pattern occurred in disks five weeks old, with further lignification of the tissues. At the site of inoculation all tissues were altered but away from this the pith and vascular tissues were most affected by the bacteria.

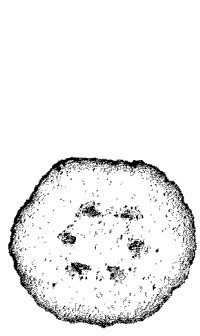
b) Medium + 0.01 ppm IAA. Disks inoculated with A. tumefaciens and grown on a medium containing 0.01 ppm IAA showed a greater proliferation of tissues from both surfaces, as compared with disks grown without added auxin (Figure 9.8b). The cortex remained unchanged by the bacteria but the vascular tissue formed a complete ring consisting mainly of small scattered xylem vessels, the majority of which lay close to the protoxylem. Proliferation from the upper surface consisted mainly of phloem and xylem elements scattered at random; below this, a ring of disorganized vascular elements was enclosed by the cortex as in the disks grown without added auxin. Throughout the pith tissue scattered tracheids and small areas of dividing cells occurred. From the lower surface the proliferation differed from that of disks grown without added auxin. The vascular bundles did not become rounded off and there was very little lignification of this lower tissue. Two or three roots emerged from between vascular bundles near the lower surface

and these generally penetrated the cortex. In contrast to disks grown without added auxin root growth was inhibited only a little when this concentration of IAA was present. In the former root primordia often did not emerge from the cortex.

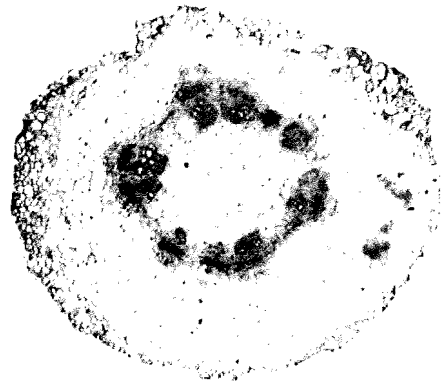
c) Medium + 0.1 ppm IAA. A variable reaction was shown by disks inoculated with A. tumefaciens and grown on a medium containing 0.1 ppm IAA. All disks produced a large amount of proliferation from the upper surface but the tracheids occurring in this were scattered amongst dividing cells (Figure 9.8c) or were organized into whorls surrounded by a cambium and phloem parenchyma (Figure 9.8d). The latter type of proliferation showed the same organization as in disks grown on media containing 0.01 ppm IAA. In disks three weeks old very little vascularization had taken place and no interfascicular cambium was formed. These disks showed the influence of the growth substance more than did disks five weeks old, which had well-developed vascular cylinders. The pith tissue showed some cell division and occasional tracheids, but the cortical tissues were comparatively little affected by either the bacteria or the auxin. An average of five or six long roots emerged from each disk. The influence of this concentration of auxin was seen most in the lower portion of the disk where lignification was inhibited.

Histology of Disks Grown in the Dark.

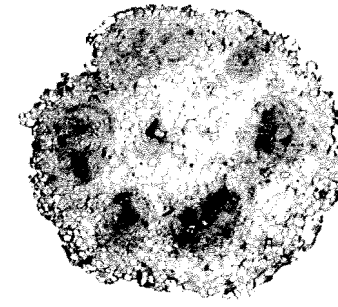
At the time of preparation of the disks and inoculation four days after germination of the seeds, the structure of sunflower hypocotyls grown in the dark was similar to that of the light-grown



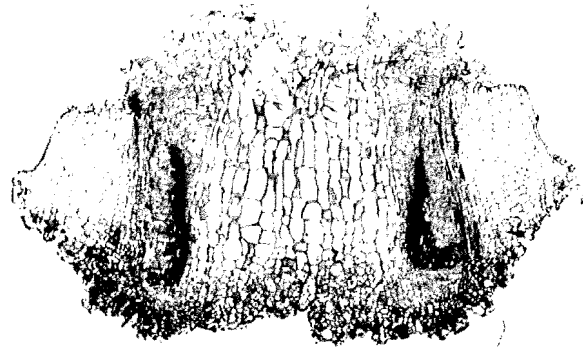
a)T.S. at time of inoculation- 4 days



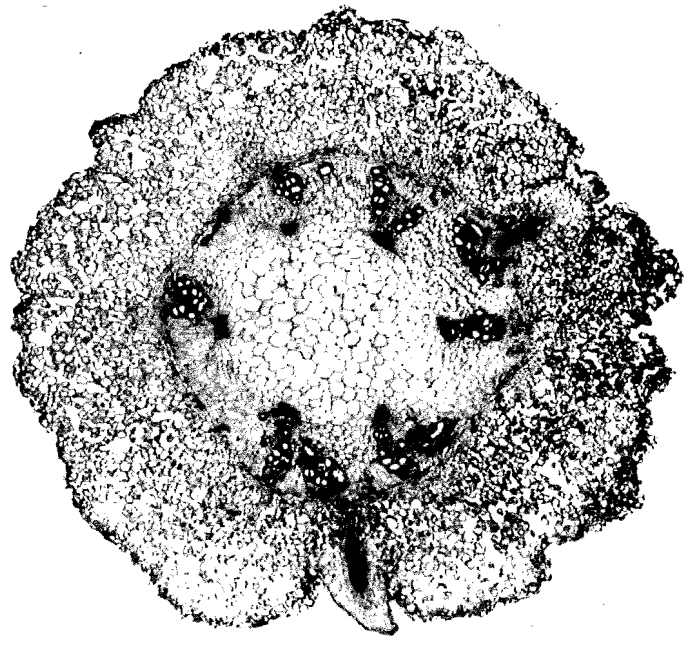
b)T.S. nine days after inoculation



c)T.S. nine days after inoculation- wound cambium



d) L.S. three weeks after inoculation



e) T. S. five weeks after inoculation

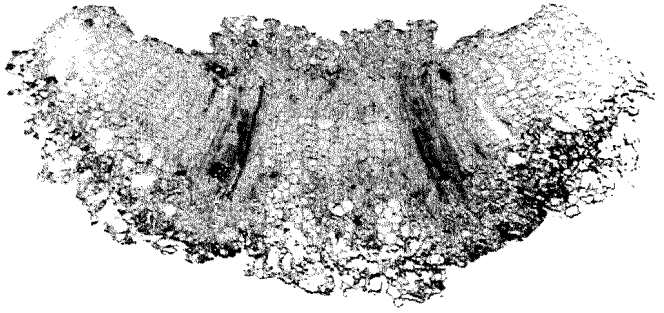
FIGURE 9.9 UNINFECTED DISKS GROWN IN THE DARK ON MEDIUM WITHOUT ADDED IAA (Mag. x 12.5)

hypocotyls (Figure 9.1b and 9.9a). The cortex was larger and the pith smaller in the dark-grown hypocotyls, but this difference was due to the size of the cells rather than to an increase in their number. Less thickening of the vascular bundles occurred in these disks. These effects can be ascribed to the etiolation of the tissues.

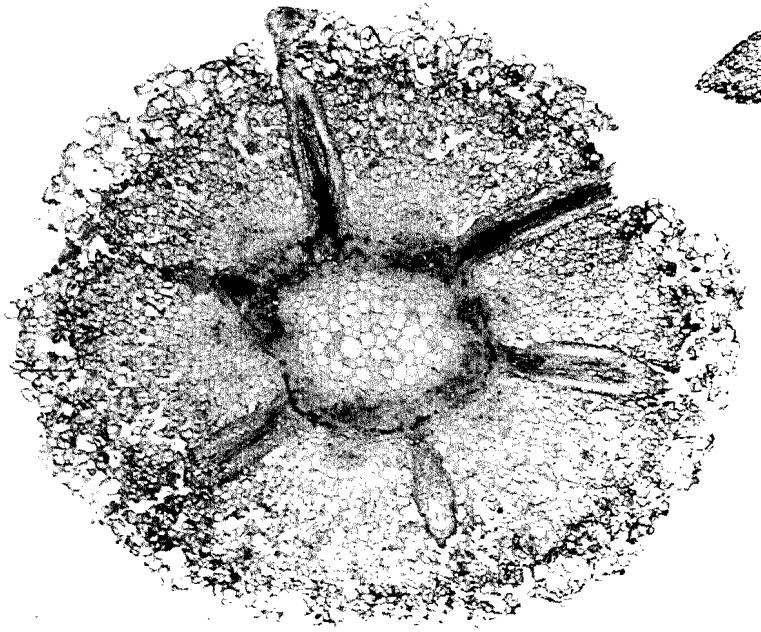
9.5 Uninfected Disks.

a) Medium without added IAA. Nine days after inoculation with sterile nutrient broth hypocotyl disks showed an expansion of both the cortical and pith parenchyma. A small interfascicular cambium linked the vascular bundles but all secondary thickening was confined to the original bundles where large vessels were scattered among the more regular xylem tissues (Figure 9.9b). The origin of a root primordium was clearly shown to be in the same position as described for disks grown in the light. A wound cambium developed at both cut surfaces surrounding the vascular bundles and extending into the pith (Figure 9.9c). No lignification was associated with this cambium. A continuation of growth and lignification of the vascular bundles occurred after three weeks. Further activity of the interfascicular cambium was evident and up to three roots emerged through the cortex. In longitudinal section (Figure 9.9d) it could be seen that a wound cambium had formed at the lower surface; this was added to mainly by tissues from the vascular cambium and parenchyma but some of the inner cortical parenchyma contributed to the proliferating tissue. At the upper surface, the vascular tissue had proliferated but no organized wound cambium was formed.

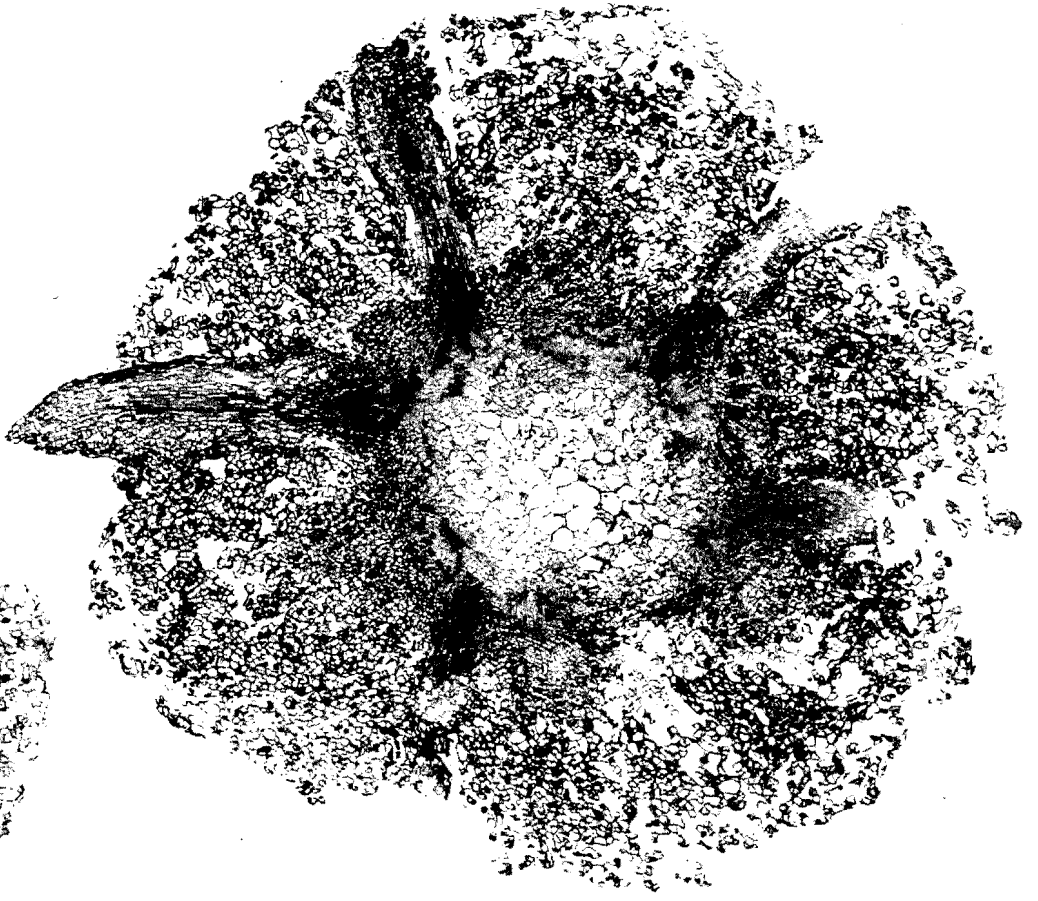
In contrast to younger disks, the cortex of disks five weeks old was of a more filamentous nature with large lacunae near the



a) L.S. fifteen days after inoculation



b) T.S. fifteen days after inoculation



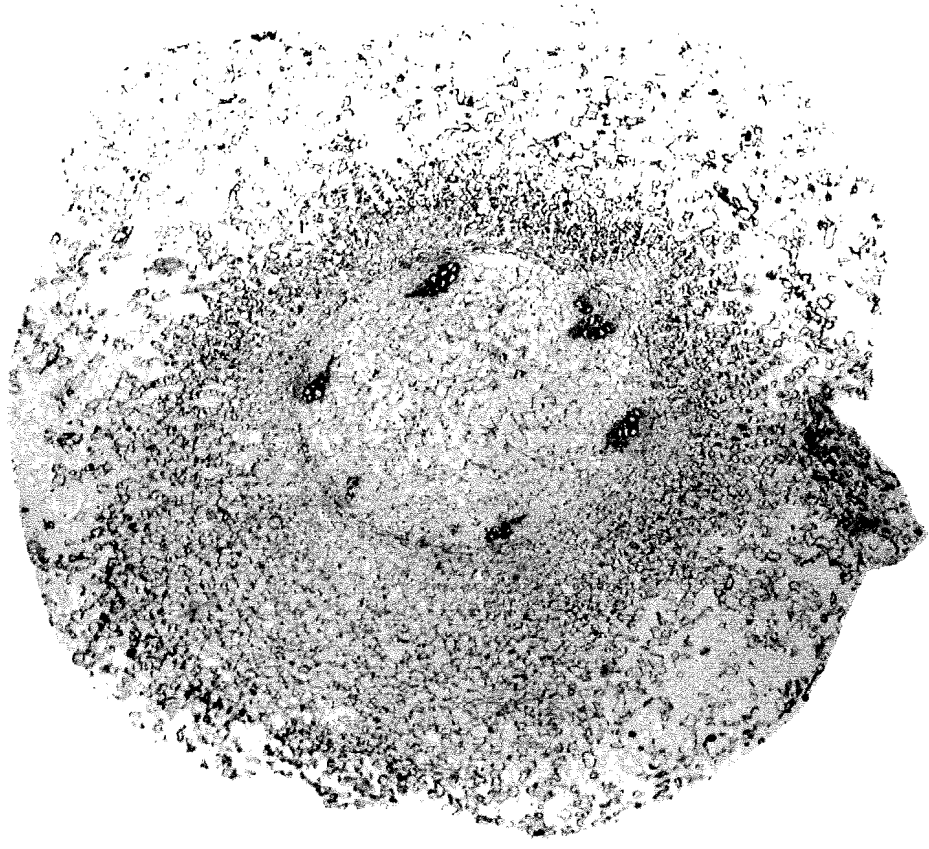
c) T.S. five weeks after inoculation

FIGURE 9.10 UNINFECTED DISKS GROWN IN THE DARK ON MEDIUM CONTAINING 0.01 PPM IAA (Mag. x 12.5)

lower wound cambium. Further lignification of the vascular tissues continued (Figure 9.9e).

b) Medium + 0.01 ppm IAA. Disks grown on a medium containing 0.01 ppm IAA showed that an increase in the expansion of the cortical tissues had occurred, caused mainly by cell enlargement and the formation of lacunae between rows of parenchyma (Figure 9.10a). Some cortical parenchyma at the base of the disk had been stimulated to divide, although no organized wound cambium was formed in this particular tissue. A few divisions were also evident higher up the cortex, and cell division had also taken place throughout the pith. The vascular bundles of all disks from two to five weeks old showed very little lignification of the xylem. An interfascicular cambium was formed and its activity resulted in the formation of up to six roots in each disk (Figure 9.10b). In these disks, the endodermis had been stimulated to divide near the vascular bundles (Figure 9.10c), so that a large quantity of parenchyma was formed between the phloem and the secretory canals. In most cases a well organized wound cambium was formed at both upper and lower surfaces, crossing the pith and encircling the ends of the vascular bundles. A large amount of parenchyma was formed from this at the upper surface, while at the lower surface, hypertrophied and hyperplastic cells were intermixed.

c) Medium + 0.1 ppm IAA. After only fifteen days' growth, disks grown on a medium containing 0.1 ppm IAA showed a massive expansion of the parenchymatous tissues which exceeded that of disks grown on a medium containing 0.01 ppm IAA. Occasional cell divisions did



T.S. three weeks after inoculation

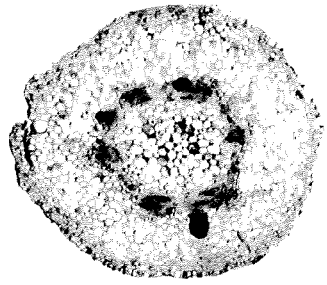
FIGURE 9.11 UNINFECTED DISK GROWN IN THE DARK ON MEDIUM CONTAINING 0.1 PPM IAA (Mag. x 12.5)

occur. In all disks examined up to five weeks of age the vascular tissue remained in the original bundles and very little secondary thickening took place; no interfascicular cambium was formed (Figure 9.11). The vascular cylinder was encircled by the endodermis which was dividing, more particularly above the vascular bundles. Many roots were formed in all the disks; these emerged through the cortex from the base of the disk after running parallel to the vascular cylinder.

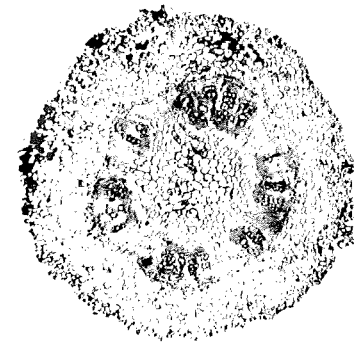
9.6 Disks Inoculated with E. coli.

a) Medium without added IAA. Disks inoculated with E. coli and grown in the dark showed one of two reactions. Either the interfascicular region was stimulated with the initiation of root primordia while the remainder of the tissues were unexpanded with little secondary growth, or the cortical cells expanded with large lacunae formed between the rows of parenchyma. This second reaction was similar to that of E. coli-inoculated disks grown in the light.

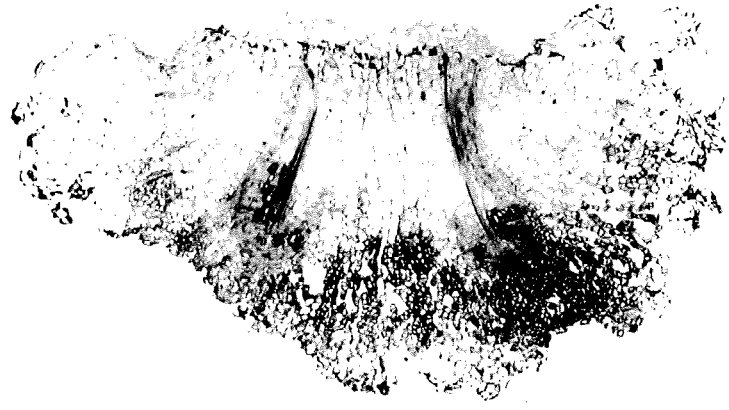
In a disk showing the first type of growth, a little expansion of the parenchymatous tissues took place after eleven days' growth (Figure 9.12a). The vascular bundles remained discrete and showed only a small amount of secondary growth, but some activity of the interfascicular region had brought about the initiation of a number of root primordia. Further growth of these primordia into the cortex appeared to be inhibited. A disk of the same type showed, after five weeks' growth, that although there was no further expansion of the cortex or pith, a wide interfascicular cambium



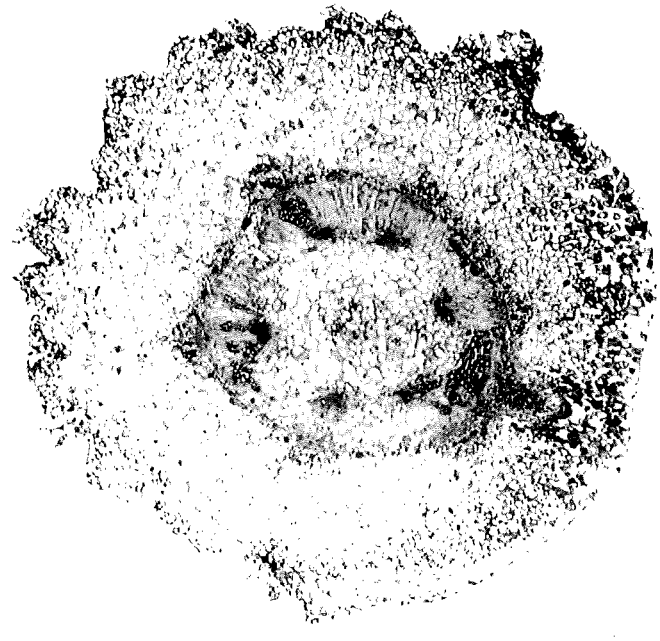
a) T.S. eleven days after inoculation



b) T.S. five weeks after inoculation



c) L.S. two weeks after inoculation



d) T.S. three weeks after inoculation

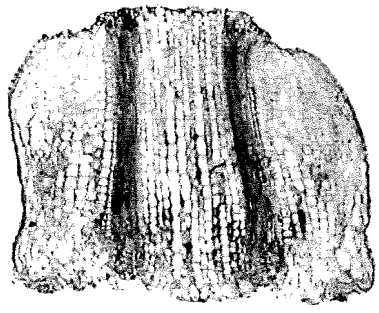
FIGURE 9.12 DISKS INOCULATED WITH E.COLI AND GROWN IN THE DARK ON MEDIUM WITHOUT ADDED IAA (Mag. x 12.5)

joined the vascular bundles. Within these bundles lignification of the xylem tissues had occurred (Figure 9.12b).

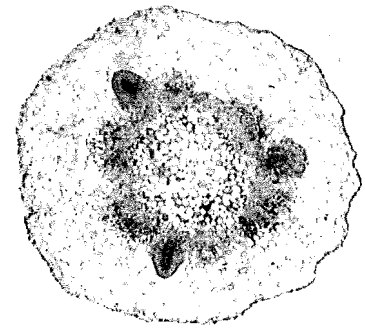
Disks sectioned after two and three weeks of growth reacted similarly to E. coli-inoculated disks grown in the light. In longitudinal section (Figure 9.12c) it could be seen that the lower part of the epidermis and outer cortex had expanded and disintegrated. A few lacunae had developed between rows of cortical parenchyma but these were not as large as in those plants grown in the light. The pith tissue had not expanded as in the controls grown without bacteria. A well-developed wound cambium was formed at the upper surface, crossing the pith, while at the lower surface the cambium was not complete, but enclosed the ends of the vascular bundles. A few scattered cell divisions occurred in the pith near the lower surface of the original tissue. The vascular tissue remained localized in the original vascular bundles which were linked by a wide interfascicular cambium. The secondary xylem was characterized by a band of recently-formed large vessels. Up to three roots emerged from each disk (Figure 9.12d).

b) Medium + 0.01 ppm IAA. As in disks inoculated with E. coli and grown on a medium devoid of auxin, those grown on a medium containing 0.01 ppm IAA showed a variable reaction. Either the cortex of the disks expanded only a little, or a large amount of expansion occurred with proliferation from the lower wound cambium. A majority of the disks reacted in the latter way.

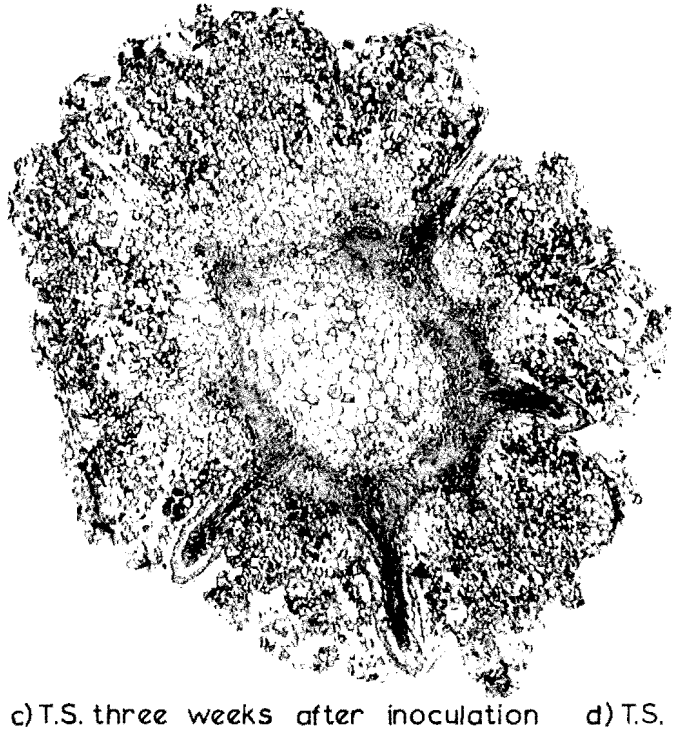
Disks sectioned after fifteen days' growth showed only a slight expansion of the cortical cells, which, at the lower surface, had started to collapse (Figure 9.13a, b). The pith parenchyma had also



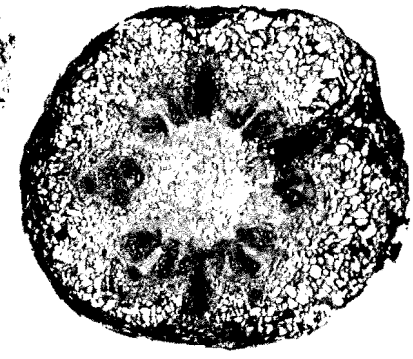
a) L.S. fifteen days after inoculation



b) T.S. fifteen days after inoculation

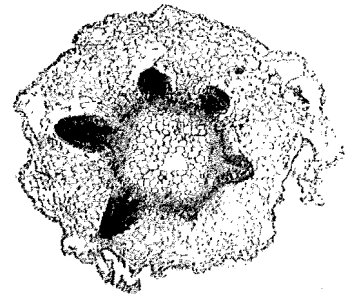


c) T.S. three weeks after inoculation



d) T.S. five weeks after inoculation

Medium + 0.01 ppm IAA



e) T.S. three weeks after inoculation - Medium + 0.1 ppm IAA

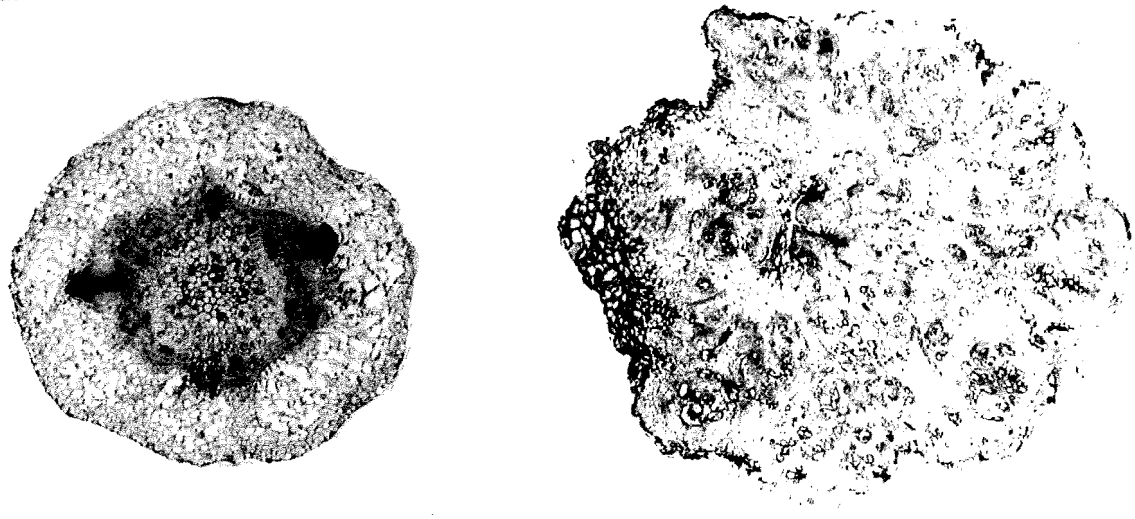
FIGURE 9.13 DISKS INOCULATED WITH E.COLI AND GROWN IN THE DARK ON MEDIA CONTAINING 0.01 AND 0.1 PPM IAA (Mag. x 12.5)

expanded only a little. No interfascicular cambium had formed and the vascular bundles themselves showed little secondary thickening. However the interfascicular region was stimulated and up to six root primordia were formed. These extended only a short way into the cortex where their growth was inhibited. In longitudinal sections no wound cambium was evident, although in transverse sections of some disks it was visible at the lower surface.

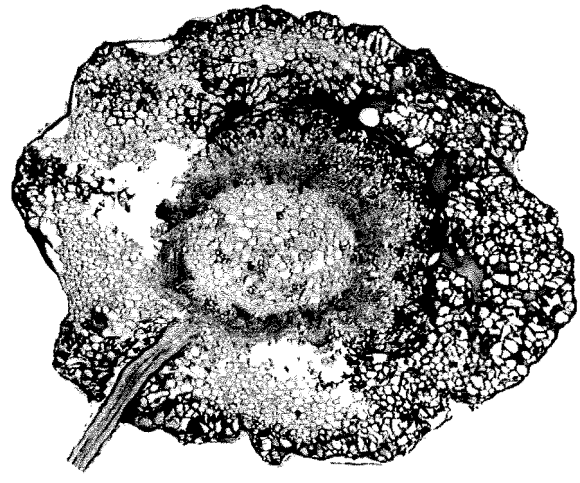
Two disks taken from different parts of the same hypocotyl reacted in completely different ways to the bacteria, after three weeks' growth. In one the structure was similar to that described for fifteen day-old disks, but in the other, expansion of the cortex had occurred; this disk was similar to those controls grown on 0.01 ppm IAA-containing medium (Figure 9.13c). With the production of six roots its growth was similar to that of disks not inoculated with E. coli.

Disks sampled after five weeks of growth were similar in form to the younger disks, showing collapse of the outer cortical tissue and hypertrophy and hyperplasia in the inner cortex. The vascular tissues in these disks had developed considerably and a large amount of secondary xylem was formed. At the lower surface, a well-defined wound cambium crossed the pith (Figure 9.13d). Root primordia were again present between the vascular bundles.

c) Medium + 0.1 ppm IAA. Growth of disks inoculated with E. coli was greatly inhibited on a medium containing 0.1 ppm IAA. The cortical and pith tissues which had started to disintegrate in disks three weeks old showed only a little expansion (Figure 9.13e). The



a)T.S. nine days after inoculation b)T.S. proliferation five weeks after inoculation
Medium without added IAA



c)T.S. three weeks after inoculation - Medium + 0.01 ppm IAA

d)T.S. three weeks after inoculation - Medium + 0.1ppm IAA

FIGURE 9.14 DISKS INOCULATED WITH A.TUMEFACIENS AND GROWN IN THE DARK ON MEDIA CONTAINING 0, 0.01 AND 0.1 PPM IAA (Mag. x 12.5)

vascular tissue had developed only a little after inoculation, but the interfascicular region had been stimulated to divide, producing up to six root primordia. Growth of these was inhibited so that roots rarely emerged through the cortex; the IAA present in the medium further influenced the roots so that they grew down through the disk. The endodermis in these disks was active, dividing above the vascular bundles.

Disks five weeks old showed slightly more lignification of the vascular tissue, but the other tissues remained the same as those three weeks old.

9.7 Disks Inoculated with A. tumefaciens.

a) Medium without added IAA. Nine days after inoculation with A. tumefaciens disks showed some proliferation from the upper surface mainly in the form of dividing cells interspersed with a few tracheids. Below this proliferation the original cortex had undergone a little expansion although most of its tissues had collapsed by the time of sectioning (Figure 9.14a). Nearer the upper surface large whorls of lignified tracheids had formed in the inner cortex as in disks inoculated with A. tumefaciens and grown in the light. Lignified xylem vessels were present near the protoxylem but vessels which were not as heavily lignified occurred between the bundles. Throughout the pith some cells had divided and in places tracheids, lacking any particular orientation, had been formed. A number of root primordia were formed in the interfascicular region near the base of the disk, but their subsequent growth through the cortex had been inhibited. Only a little proliferation occurred at the

base of the disk, in contrast to the upper surface.

In disks three weeks old further proliferation from the upper surface had occurred; large lobes of parenchyma and phloem elements enclosed irregular cambia and nests of tracheids. The structure of the original part of the disk as described for nine day old infected disks remained the same, but up to eight root primordia were formed. This increase above the usual maximum of six occurred when more than one primordium was formed between any particular pair of bundles.

Disks five weeks old showed a large amount of proliferation from both surfaces (Figure 9.14b). The original vascular tissue was largely disrupted by this proliferation and whorls of tracheids had formed close to the vascular bundles. Some of the roots in these disks penetrated the cortex, overcoming to a certain extent the usual inhibition of growth acting after their initiation.

b) Medium + 0.01 ppm IAA. On a medium containing 0.01 ppm IAA, disks inoculated with A. tumefaciens showed a large amount of proliferation from the upper surface, either in the form of large lobes of parenchyma and cambia enclosing tracheids, or as parenchyma interspersed with tracheids and dividing cells. At the base of the disk the proliferation consisted mainly of parenchymatous tissue with occasional tracheids. A number of root primordia were formed between vascular bundles at the base of the disk examined; in some cases these roots had grown through the proliferating tissue at the lower surface, but usually their growth was inhibited (Figure 9.14c).
As in the cortex of disks grown without added auxin, expansion was completed after three weeks and some collapse and disintegration of

the tissues occurred. At the top of the disk near the proliferating tissue whorls of tracheids and unorganized cambia occurred in the inner cortex. The vascular bundles were joined by an interfascicular cambium, derivatives of which had become lignified producing an almost continuous band of tracheids and vessels. Throughout the pith dividing cells and small nests of tracheids were present. Different reactions to the bacteria and growth substance were evident in different disks. In some, a large amount of secondary thickening occurred but in others little development took place. Generally there seemed to be more lignification in these disks than in those grown without added auxin.

c) Medium + 0.1 ppm IAA. All disks inoculated with A. tumefaciens and grown on a medium containing 0.1 ppm IAA reacted similarly. Proliferation at the upper surface in three week-old disks was mainly parenchymatous; occasional tracheids and dividing cells did occur. Whorls of tracheids were more frequent in the proliferating tissue of disks five weeks old. Proliferation from the lower surface consisted, in all cases, of parenchyma cells through which root tips passed. The roots ran parallel to the main axis and were similar to those produced by control and E. coli-inoculated disks grown on a medium containing the same concentration of auxin. Throughout the cortex and pith some cell divisions were visible but no whorls of tracheids occurred (Figure 9.14d). The original vascular tissue showed only a little growth, with some lignification of xylem vessels. A small but not very active interfascicular cambium was formed between vascular bundles.

9.8 Comparative Anatomy of Sunflower Hypocotyl Disks, Treated with sterile medium, E. coli, or A. tumefaciens and Different Concentrations of IAA.

Disks Grown in the Light.

a) Growth on Medium without added IAA. In hypocotyl disks inoculated with sterile synthetic medium some expansion of the parenchyma tissues occurred during five weeks of growth. In contrast to this, disks inoculated with E. coli showed a massive expansion of the cortical parenchyma. In both treatments the epidermal cells were unaffected, but in the presence of the bacteria they were pushed from their normal position by the internal expansion. In uninfected disks an interfascicular cambium was formed between vascular bundles contributing mainly parenchyma cells; the bundles themselves had undergone some lignification. A wound cambium developed at both cut surfaces from the vascular cambium, parenchyma, and pith, and produced mainly parenchyma cells. Disks inoculated with E. coli followed this general pattern but increased lignification of the vascular tissues and wound tracheids inside the lower wound cambium occurred. This cambium cut off more parenchyma to the outside than in the uninfected disks. While the uninfected disks generally produced no roots, and at the most, two roots, disks inoculated with E. coli regularly produced up to five roots.

The crown gall bacterium influenced hypocotyl disks in quite a different way from either E. coli, or IAA at the concentrations used. The external morphology showed that the disks remained

compact, the main proliferation arising at the upper surface from the stelar tissues, rather than from the cortex. The original tissues of the disks were influenced far more by the bacteria than in the two cases just compared. The inner cortex was stimulated to divide producing tracheids enclosed by unorganized cambia and phloem parenchyma. In some cases the outer cortical tissues divided, but generally only a little expansion occurred and the tissues disintegrated. In the pith, cell division and formation of tracheids was also stimulated. The vascular tissues also showed the influence of the bacteria with an increase in lignification compared with the two previous treatments. In A. tumefaciens-inoculated disks three or four root primordia were initiated but their further growth was retarded or completely inhibited; only occasional roots emerged through the cortex. The nature of the proliferation from the wound cambia also differed in disks inoculated with A. tumefaciens. The bulk of growth occurred at the upper surface where whorls of tracheids were mixed with unorganized cambia and phloem parenchyma.

Although E. coli increased the amount of lignification in vascular tissues its main influence was in the expansion of the lower cortical tissues, the stimulation of root production near the base of the disk, and the increased proliferation from the lower wound cambium. The bacteria (or products formed by them) apparently passed through the disk to exert an influence mainly at the lower surface. A. tumefaciens in contrast, had most effect in the upper regions of the disk, producing a large amount of relatively

well-developed tissues in an unorganized mass. The influence of the bacteria was further in evidence in the original tissues of the disk with the stimulation of cell division and tracheid development near the upper surface, and the inhibition of root growth after initiation at the lower surface.

b) Growth on Medium + 0.01 ppm IAA. Disks inoculated with E. coli and grown on a medium containing 0.01 ppm IAA showed a greater expansion of the cortical tissues than uninfected disks. The characteristic expansion and disintegration of the cortex and of the parenchyma derived from the lower wound cambium, was not inhibited by the presence of the auxin. The bacteria appeared to exert a greater organizing effect on the wound cambia at both surfaces than was achieved by the growth substance alone. The amount of lignification of the xylem tissues was similar in both the uninfected and E. coli-inoculated disks, although a greater number of larger vessels occurred in the latter case. Cell divisions were more frequent in the pith and the cortex in both these cases, as compared with disks grown without added auxin. Up to four or five roots were produced in disks inoculated with the bacteria while in uninfected disks only two or three roots were formed. A stimulus in addition to the growth substance may have been acting on the interfascicular region to produce more roots in E. coli-inoculated disks. Histologically there were fewer distinctions between uninfected and E. coli-inoculated disks grown on a medium containing this concentration of IAA than were noted for the same disks grown on a medium without the auxin.

Inoculation with A. tumefaciens of disks grown on a medium

containing 0.01 ppm IAA resulted in much proliferation from the upper surface and some proliferation from the lower wound cambium. The general structure of the disks was similar to that of disks grown without added auxin. Scattered tracheids and dividing cells occurred throughout the pith and unorganized whorls of tracheids were present in the inner cortex. The disks did differ, however, at the lower surface, from those grown without auxin. There was very little lignification of the vascular tissues and the proliferating wound cambium produced parenchyma.

All three treatments showed the effects of the auxin at the lower surface, in the first two cases with the increased proliferation from the wound cambium, and in the third with the decreased lignification. The number of roots formed in disks inoculated with A. tumefaciens, E. coli and sterile medium was increased proportionately from the number formed when no IAA was present in the medium. Addition of the growth substance decreased the differences between the control and E. coli-inoculated disks, but had little effect on disks inoculated with A. tumefaciens, serving to emphasize the differences between the two bacterial treatments.

c) Growth on Medium + 0.1 ppm IAA. Histologically, there was little to distinguish disks inoculated with E. coli from those inoculated with synthetic medium, when grown on a medium containing 0.1 ppm IAA. An increased expansion of the cortex occurred with the former treatment but in both cases the same number of roots was usually formed. A more regular wound cambium developed in E. coli-inoculated disks and secondary thickening was inhibited a little as

compared with the uninfected disks.

Disks inoculated with A. tumefaciens again proliferated from the upper surface with the formation of either scattered tracheids and dividing cells, or a more organized type of proliferation. The added auxin was effective in limiting lignification in the disks, particularly near their bases and in overcoming the inhibition of root growth to a certain extent, so that five or six roots emerged from the cortex.

On this medium the effects of the bacterial treatments were overshadowed by the response of the disks to the auxin. This was particularly the case with E. coli; inoculation with A. tumefaciens produced fewer irregular areas of cell division and tracheid production within the original tissue of the disk.

Disks Grown in the Dark.

d) Growth on Medium without added IAA. The growth of disks inoculated with E. coli was generally inhibited in comparison with that of uninfected disks. Parenchyma tissues of the uninfected disks expanded and some lignification of the vascular tissue occurred, together with the development of interfascicular cambium. Wound cambia formed at both surfaces. Uninfected disks produced up to six roots each.

In most disks inoculated with E. coli little expansion of the cortex and pith occurred. The interfascicular cambium developed but was less active than in the uninfected disks and there was less lignification of the vascular tissues. As with the uninfected

disks five or six root primordia were initiated but growth of these through the cortex was inhibited. Some disks inoculated with E. coli showed more expansion of the cortical tissues than in the uninfected disks, as was observed in disks grown in the light. There was little difference in the size and lignification of the vascular tissues of these disks as compared with the uninfected disks, but the lower wound cambium was not as well organized, although it produced more parenchyma tissue.

Disks inoculated with A. tumefaciens reacted similarly to those grown in the light. Only a little expansion of the cortex occurred before some of the tissues collapsed. Near the upper surface whorls of tracheids were formed outside the vascular bundles.

As with disks grown in the light, a basic distinction was evident between the two bacterial treatments. In the majority of cases inoculation with E. coli caused an inhibition of growth compared with uninfected disks. A. tumefaciens again caused proliferation at the upper surface and division and lignification in the original tissues.

e) Growth on Medium + 0.01 ppm IAA. As in the disks just described, those inoculated with E. coli and grown on a medium containing 0.01 ppm IAA mainly showed an inhibition of growth as compared with the uninfected disks. While little development of the vascular tissues occurred in the uninfected disks, those inoculated with E. coli showed considerable thickening after five weeks, although there was only slight expansion of the parenchyma.

The formation of root primordia was stimulated in both treatments, but in the presence of the bacteria further growth was frequently inhibited. Proliferation of the endodermis, apparent in uninfected disks, was also generally inhibited by the presence of E. coli. Some disks inoculated with this strain of bacteria did expand, and were very similar in form to the uninfected disks. Both uninfected and E. coli-inoculated disks grown on this medium were larger than their counterparts grown without added auxin.

In disks inoculated with A. tumefaciens the upper surface proliferated and the whorls of tracheids present in the inner cortex were similar to those in disks grown without added auxin. The IAA was effective at the lower surface in stimulating parenchyma production from the wound cambium. In the disks observed, the original vascular tissues generally become more lignified than in the uninfected disks although this may not have been the usual reaction since lower concentrations of IAA appeared to be more reactive in dark-grown disks. Roots were formed near the lower surface and these often grew parallel to the vascular cylinder before emerging through the lower proliferating tissue. This growth form was usual only in disks grown on media containing 0.1 ppm IAA and reflected the influence of the auxin, acting in conjunction with the bacterial growth substances.

f) Growth on Medium + 0.1 ppm IAA. The growth of disks inoculated with E. coli and grown on a medium containing 0.1 ppm IAA was inhibited to a greater extent than that of disks grown without added auxin in the presence of the bacteria. This limited growth

contrasted markedly with the massive expansion of parenchyma in uninfected disks. Even less lignification of the vascular tissue occurred in E. coli-inoculated disks than in the uninfected disks. In both treatments initiation of root primordia was stimulated, but their further growth was inhibited in bacterial-inoculated disks; in the uninfected disks roots grew parallel to the vascular cylinder before emerging. In both treatments the endodermis divided but more tissue was produced from it in the uninfected disks.

Disks inoculated with A. tumefaciens showed the influence of the bacteria in the upper tissues where proliferation occurred, but much lignification of the vascular tissue was prevented by the presence of auxin. The amount of cell-division and tracheid formation in the pith and cortex was also reduced by the presence of IAA in the medium. Only a small expansion of the original tissues occurred and it would appear that as with E. coli, A. tumefaciens acted synergistically with the IAA to give a marked reduction in the final size of the disks, as compared with these disks, grown without auxin.

9.9 A Comparison between Disks Grown in the Light and the Dark.

Taking the different treatments collectively, greater lignification occurred and more phloem fibres were formed in light-grown disks, while in disks grown in the dark, division of the endodermis was stimulated.

Uninfected disks grown without added IAA produced more roots when grown in the dark, although the remaining structure was similar to that of disks grown in the light. The IAA present in

the medium was more effective in the dark than in the light in inhibiting lignification of vascular tissues. At the highest concentration observed, uninfected disks grown in the dark were more affected by the auxin than light-grown disks; this was evident from the characteristic growth of the roots downwards through the tissue in the disks grown in the dark. In both cases the same amount of expansion occurred increasing with increasing concentration of auxin.

E. coli-inoculated disks grown in the light and the dark showed the greatest differences of all treatments. In the light, expansion of the cortex was stimulated and many long roots were formed. In the dark little expansion of the parenchyma occurred, and although up to six root primordia were initiated, their further growth was inhibited. It is possible that in all dark-grown disks root formation was stimulated, but E. coli inhibited any further growth of the root primordia, as well as of the cortical tissues. In the light, uninfected disks formed only occasional roots whereas E. coli-inoculated disks regularly produced a number. This differing behaviour in the light and the dark is not consistent with the hypothesis that E. coli liberates a growth substance similar to IAA.

With increasing concentrations of IAA, disks inoculated with E. coli reacted differently in the light and the dark. In the light they became almost indistinguishable from uninfected disks when grown on a medium containing 0.1 ppm IAA, but in the dark the presence of auxin at this concentration emphasized the inhibiting

action of the bacteria.

In A. tumefaciens-inoculated disks the basic structure remained the same in the light and the dark and was modified only a little by the inclusion of IAA in the medium. The auxin was generally more effective in inhibiting lignification of the original tissues in the dark than in the light.

9.10 Discussion.

The present work suggests that in sunflower hypocotyl disks grown with different concentrations of IAA certain changes occurred that were similar to the changes induced by the application of auxin to other plant tissues. However, some reactions of the sunflower tissue did not correspond with reported changes induced by IAA application. For example, Snow (1935) found that treatment of sunflower hypocotyls with auxin resulted in increased secondary xylem formation with associated secondary thickening, but in the present work decreased lignification occurred when uninfected disks were grown on all of the IAA media. De Ropp (1947a) reported that when segments of the first internode of sunflower stem were grown on media containing 10 ppm IAA the cambium proliferated with the formation of loosely connected tissue. Tissue of this type was observed in hypocotyl disks grown with 0.1 ppm IAA, but it was formed from the wound cabium at the surface of the tissue in contact with the medium, rather than from the vascular cambium as described by de Ropp. De Ropp also reported the presence of xylem-like tissue between the vascular bundles when stem tissue pieces were grown on medium containing 0.1 ppm IAA. In the present work, however, an

interfascicular cambium was observed to develop in this region which gave rise to parenchyma, particularly in light-grown disks. The endodermis of hypocotyl disks grown in the dark was stimulated when IAA was included in the medium. Blum (1941) found that auxins stimulated growth of the endodermis in stems of sunflower plants, while Kraus et al (1936) and Palser (1942) showed that the endodermis in red kidney bean and Vicia faba was one of the tissues most affected by the application of IAA.

Root growth in uninfected hypocotyl disks was increased when 0.01 ppm IAA was included in the medium. With 0.1 ppm IAA, growth of roots was stimulated compared with disks grown without IAA, but the hypocotyl tissues, particularly when grown in the dark, became hyperhydric. At this concentration of IAA, many of the roots tended to be short and lacked the well-defined structure of roots formed with the lower concentrations of IAA. Cortical tissues of these roots became filamentous and large lucanae were formed. These observations are in accord with Gautheret's (1959) report that sunflower tissue in culture first formed roots and later became hyperhydric with increasing concentration of IAA.

All hypocotyl disks examined, both infected and uninfected, formed roots in the interfascicular region. In other stem tissues, the origin of roots induced by the application of auxin has been described as mainly from the interfascicular region, although phloem, xylem, cambium, endodermal and pith tissues have also been reported as contributing some tissues to the developing structures (Blum 1941, Kraus et al 1936, de Ropp 1947a).

The differences in fresh weights of uninfected disks grown under different light conditions and on media with varying concentrations of IAA, as described in Chapter 5, are attributable to the changes in structure of the hypocotyl disks. Less lignification occurred in dark-grown tissues as compared with those grown in the light, and the cells were larger, so that the weights of disks in the light were accordingly heavier. This difference caused by the light treatment was evident when disks were grown on all media. Studies of the fresh weights and gross morphology of uninfected hypocotyl disks showed that the same concentration of IAA was more active in influencing the structure of dark-grown than light-grown disks. From the histological observations, IAA was found to be more effective in inhibiting lignification in the vascular tissues of dark-grown disks, and these showed more hyperhydric tendencies than disks grown in the light on medium containing 0.1 ppm IAA. The cortical cells were larger in the dark-grown disks, although there was little difference in the diameters of disks grown under the two conditions.

Frequently the growth of tissues infected with A. tumefaciens has been likened to that induced in tissues treated with auxins (Thomson 1945, Braun and Stonier 1958). In the present work, however, the callus tissue induced by this bacterium at the wounded surface contained vessels, often organized into well-defined whorls which were remote from the wound cambium. In some cases a second row of vascular bundles was formed outside the vascular cylinder in the cortex of the original tissues. This peculiar type of growth

was not induced by the aplication of IAA and was found only in disks inoculated with A. tumefaciens. Vascular bundles of this nature were described by Kraus (1941) as forming in red kidney bean to which tryptophane had been applied, and by Palser (1942) who found them after treating the stem of Vicia faba with IAA. The action of A. tumefaciens in stimulating plant growth was most evident at the surface to which the bacteria were applied and few changes were evident in the tissues in contact with the medium and remote from the site of inoculation. By contrast, tissues at the upper surface of uninfected disks as well as those in contact with the medium were stimulated by the presence of IAA. The applied growth substance apparently moved further within the plant tissues than the bacteria or the stimulus resulting from their presence. This is contrary to de Ropp's (1948b) experiments showing movement of A. tumefaciens within segments of sunflower stem tissue. He found that the bacteria could travel through segments 9 mm long within four minutes. In the present work the bacterial colonies were observed to form a layer about the base of disks after inoculation. In the absence of proliferation from this surface, it was assumed that the bacteria were not present while the wounded cells were in a conditioned state, and tumour formation was not induced.

When hypocotyl disks inoculated with A. tumefaciens were grown on media containing IAA, there was a decrease in the amount of lignification of the tissues. Struckmeyer et al (1949) found that in pieces of bacteria-free crown gall sunflower tissue grown on medium containing high concentrations of IAA, fewer vessels

were formed than when no IAA was present. In this respect the crown gall callus tissues and the infected hypocotyl disks reacted in the same way.

Few attempts have been made to grow tissues in which A. tumefaciens is present (Gautheret 1959), so that most work with crown gall tissue has been carried out using cultures of bacteria-free tumour tissue. Although Braun (1953) states that no roots are formed in fully transformed crown gall calluses, there have been a few reports of root initiation in tissues of crown gall origin (Gautheret 1959). It is evident that the combination of factors necessary for root initiation is not usually present in crown gall callus. In the present work roots were formed in the original tissues of hypocotyl disks infected with A. tumefaciens but these grew only a little after initiation and the tips became blackened. The bacteria appeared to have an inhibitory effect on the continued growth of roots after their initiation; this inhibition was overcome by the addition of IAA to the medium. Reference was made in Chapter 5 to the hyperauxinic nature of crown gall tissues, and it was considered surprising that the addition of IAA to such tissues should stimulate root growth. From the present observations of the localized activity of the bacteria, it is possible that increased growth substance metabolism was confined to a small area and did not markedly influence the reactions of tissues to the application of IAA at some distance from this area. This contrasts with the reports of secondary tumour formation at some distance from the primary tumour in sunflower. For this to occur some stimulus must move considerable distances through the tissue.

From these investigations into the histology of sunflower hypocotyl disks, it is evident that the action of A. tumefaciens is quite different from that of IAA in the concentrations used.

As with uninfected disks, changes in the cellular composition of hypocotyl disks inoculated with A. tumefaciens were correlated with changes in the fresh weights. The weights of these disks were little influenced by the addition of IAA to the medium, except at the highest concentrations. When such disks were grown on media containing 0.01 or 0.1 ppm IAA, less lignification occurred in the original tissues, although some vessel elements were formed in the proliferating tissues. Increased root formation and proliferation may have balanced the decrease in lignification, thus accounting for the small change in weights. Since the structure of light and dark-grown disks inoculated with A. tumefaciens was very similar, the fresh weights of these disks varied only slightly.

In E. coli-inoculated disks, the tissues formed were quite different from those found in hypocotyl disks inoculated with A. tumefaciens. In the light the bacteria induced cell enlargement and division in tissues remote from the site of inoculation, unlike disks inoculated with A. tumefaciens where most change was observed in the tissues at the site of inoculation. The cells which were stimulated to expand frequently collapsed. Root formation was also stimulated in light-grown hypocotyl disks infected with E. coli. Because of this stimulation of cell division and root formation, fresh weights of infected disks were

higher than those of uninfected disks. The roots contributed much more to the increase in weight than in A. tumefaciens-inoculated disks where few roots were formed and the fresh weight was more a measure of the actual proliferation.

The addition of 0.01 ppm IAA to the medium on which the infected disks were grown did not eliminate the expansion and collapse of cells at the lower surface. In uninfected disks grown on this medium, cells at the lower surface expanded less than in E. coli-inoculated disks, and this was not followed by collapse. Again the increased cell division and expansion was reflected in the greater weights of these disks as compared with uninfected hypocotyl disks. With increase in the concentration of IAA to 0.1 ppm, E. coli-inoculated disks could not be distinguished histologically from uninfected disks, and their fresh weights were also similar.

In the dark, the usual growth form of E. coli-inoculated hypocotyl disks was quite different from that of similarly treated light-grown disks. Numbers of roots were initiated but their further growth was inhibited. The outer tissues of the cortex showed little growth and frequently broke down. To a limited extent, addition of 0.01 ppm IAA to the medium overcame the inhibition of growth induced by the bacteria, but with higher concentrations it was more marked.

Since some growth occurred in disks inoculated with E. coli, an increase in fresh weight was recorded at the time of harvesting. The high dry weight to fresh weight ratio of E. coli-inoculated

disks grown in the dark on medium without added IAA, was most noticeable, and could be attributed to the increased lignification and the number of root initials observed in these disks.

The action of E. coli on sunflower tissues does not appear to be similar to that of other bacteria, whose effects on plant tissues have been investigated. This study has shown that E. coli reacts in quite a different manner from A. tumefaciens. The only non-pathogenic bacteria which have been studied for their histological effects on plant tissues have been Bacillus megaterium and Azotobacter chroococcum (Fallot 1964). Jerusalem artichoke tissues infected with B. megaterium showed an increase in the number of vascular bundles produced while cork cambium and scattered tracheids were formed. However, less callus was produced than when these tissues were treated with 1 ppm IAA. When artichoke tissues were inoculated with A. chroococcum, Fallot found that the tissues reacted in a manner similar to the application of IAA.

E. coli acts on tissue growth in a different way from either the wound tumour virus where more cell division than enlargement occurs, or the genetic tumours of Nicotiana hybrids in which these tissues become relatively well organized.

It is evident that the effects of E. coli on the growth of sunflower hypocotyl tissues cannot be equated with any known agent of abnormal growth. A combination of auxins and cytokinins may be able to induce growth in sunflower tissues similar to that described for E. coli. However this would be difficult to demonstrate as the number of these substances available and the different concentrations which could be used are almost limitless.

CHAPTER TEN

DISCUSSION AND CONCLUSIONS

This work was a preliminary investigation of the ability of Escherichia coli to induce proliferation in sunflower hypocotyl tissue, a phenomenon first described by Philipson and Sheat (1963). The exploration was largely descriptive but from the results some insight into the nature of the stimulus and its mode of action has been possible.

The tests Philipson and Sheat made using decapitated seedlings were repeated and a definite but variable response to inoculation with E. coli was found. This was an intensification of the wound response at the cut surface of the hypocotyl as determined from the gross morphology. In these experiments it was immediately apparent that the callusing induced by E. coli was quite different from that formed by Agrobacterium tumefaciens. With A. tumefaciens, the response was much greater than could be attributed to wounding, and was of a different nature. Decapitated seedlings inoculated with Bacillus megaterium did not callus and it is evident that this bacterium is reactive only in certain tissues. Fallot (1964) induced marked proliferation in tissues of vine and Jerusalem artichoke with B. megaterium, but growth was inhibited in bacteria-free crown gall tissues and in excised roots of flax. Since B. megaterium did not initiate proliferation in sunflower tissue, its mode of action was considered to differ from that of E. coli.

For all bacterial treatments the growth of decapitated seedlings was observed in different light conditions. Hypocotyls inoculated

with E. coli gave no consistent reactions in the light and the dark, but it was evident from the small numbers of hypocotyls inoculated with A. tumefaciens that more large galls were formed when the decapitated seedlings were grown in light of high or low intensity than in the dark. This was the only occasion in the experimental work when tissues inoculated with A. tumefaciens showed different growth in the light and darkness. The difference may have been caused by a specific effect of light on the tissues adjacent to the galls rather than on the infected tissues. However, light has been shown to increase the growth of bacteria-free crown gall tissues at temperatures above 26°C (de Capite 1955).

Once it had been established that E. coli did in fact affect the growth of sunflower tissues, a more suitable assay was required than the highly variable decapitated seedlings previously used. The disks of hypocotyl tissue provided this means of assay.

The large differences between uninfected and E. coli-inoculated disks grown under various light conditions remained constant throughout most of the period of experimental work. When disks were grown under conditions of high light intensity, those inoculated with E. coli proliferated from the lower surface and formed many long roots. This did not occur with uninfected disks. In light of low intensity, E. coli-inoculated disks generally showed only a little growth and short roots were formed while uninfected disks were much larger with many long roots. Even less growth occurred in E. coli-inoculated disks grown in the dark, while uninfected disks were similar to those grown in the low light. In all light

conditions disks inoculated with A. tumefaciens produced a large amount of proliferation from the upper surface and formed short roots with blackened tips.

Compared with decapitated hypocotyls, hypocotyl disks grown under different light conditions responded in quite a different way to inoculation with E. coli. The growth of uninfected and E. coli-inoculated disks was distinct and differed in turn from that of tissues inoculated with A. tumefaciens. Disks inoculated with B. megaterium did not proliferate under any of the light conditions, verifying that the action of this bacterium on sunflower tissues was different from that of E. coli.

The dry weight to fresh weight ratios of disks showed that those inoculated with A. tumefaciens and grown in all light conditions absorbed the greatest amounts of water. For disks grown in the high light those inoculated with E. coli or nutrient broth showed similar amounts of water uptake and cell division, but in the low light and darkness least water uptake occurred in E. coli-inoculated disks. These disks increased in size and histological studies showed that lignification and cell division occurred rather than expansion, confirming the observation of a low dry weight to fresh weight ratio for these tissues.

These preliminary experiments with hypocotyl disks showed that some interaction occurred between inoculation with E. coli and the light conditions under which inoculated disks were subsequently grown. There was no such interaction in disks inoculated with A. tumefaciens. The site of response to inocu-

lation with E. coli and A. tumefaciens also differed, confirming that the stimulus from E. coli which induced proliferation was of a different nature from that produced by A. tumefaciens.

The experiments with older sunflower plants showed that inoculation of the hypocotyls with E. coli induced the formation of more roots than when hypocotyls were treated with sterile nutrient broth or synthetic medium. In neither case was proliferation from the wound induced, whereas hypocotyls inoculated with A. tumefaciens formed large tumours and numbers of roots were produced. E. coli could not therefore stimulate proliferation but could induce root formation near the wounded tissue to which it was applied. Since auxins stimulate root formation and E. coli is known to synthesize IAA in the presence of tryptophane (Hopkins and Cole 1903) it was probable that an auxin was formed by the bacteria in association with the sunflower tissues.

This supposition was tested by comparing the growth of E. coli-inoculated disks with uninfected disks grown on media containing varying concentrations of IAA. The experiment showed that the reactions of hypocotyl disks to inoculation with E. coli were different from the response of the tissues to IAA. The bacteria induced different growth forms in the light and the dark, while uninfected disks grown on media containing IAA were similar in both light conditions.

In the light, disks inoculated with E. coli and grown without IAA were very similar to uninfected disks grown on medium containing 0.01 ppm IAA. In both cases proliferation was induced at the lower

surface and root formation was promoted. The histological studies of disks inoculated with E. coli and grown on medium without IAA and of uninfected disks grown on media containing low concentrations of IAA, showed that with both treatments the cells at the lower surface proliferated and expanded, but in E. coli-inoculated disks a greater amount of proliferation occurred and the cells later collapsed. The distinction between the action of E. coli and IAA remained when the infected disks were grown on medium containing 0.01 ppm IAA. The histological studies therefore showed that for light-grown disks the action of E. coli could not be equated with that of low concentrations of IAA.

The reaction of hypocotyl disks grown in the dark to inoculation with E. coli could not be identified with any known properties of IAA. The bacterial inhibition of growth was overcome to a limited extent by the addition of 0.01 ppm IAA to the medium, showing that the effects of E. coli were not equivalent to an excess of auxin. A histological examination of E. coli-inoculated disks grown in the dark showed that root initiation was stimulated but subsequent growth of the newly formed roots was inhibited. Some cell division and xylem lignification occurred in these disks although there was little cell enlargement.

Hypocotyl disks were also inoculated with A. tumefaciens and grown under comparable conditions to those infected with E. coli. Unlike E. coli-inoculated disks grown in the light the addition of IAA to disks inoculated with A. tumefaciens had little effect on their growth.

The distinctions between growth of disks inoculated with *E. coli* and *A. tumefaciens* were examined in detail in the histological studies. While the stimulus from *E. coli* appeared to pass through the tissues to the base of the disk where most cell enlargement and division were induced, disks inoculated with *A. tumefaciens* showed the effects of the bacteria at the upper surface. Little stimulus appeared to pass through these disks and in fact increased rooting occurred at the lower surface when 0.01 ppm IAA was added to the medium. Both *E. coli* and *A. tumefaciens* are motile and de Ropp (1948b) has shown that the latter bacteria can travel through a segment of sunflower stem 9 mm long in as short a time as four minutes. No observations on the rate of movement could be made in the present work but it was apparent that although *A. tumefaciens* was present at the lower surface of the disks it was unable to induce as much proliferation as at the upper surface. In hypocotyl disks the movement of the bacteria may have been slow so that they would not have reached the lower surface while the tissues were conditioned. In the light of de Ropp's work the difference in the site of action between *E. coli* and *A. tumefaciens* cannot be attributed to their relative movement through the tissues.

The tissues formed as a result of inoculation with *A. tumefaciens* were also different from those induced by *E. coli*. With the crown gall bacterium the proliferating tissue contained numerous wound tracheids sometimes organized into whorls, while mainly parenchyma was formed in tissues inoculated with *E. coli*.

The action of A. tumefaciens on plant tissues has frequently been equated with that of auxins, particularly IAA, but these histological studies were in agreement with de Ropp's (1950) findings and showed that they could not be so equated.

In the formation of crown galls it has been shown that cells must be conditioned by wounding before they can become transformed into tumour cells. This fact led to the investigation of whether E.coli acted in a like manner and required freshly wounded cells before proliferation could be initiated. In light-grown disks the greatest response to the bacteria occurred when they were inoculated immediately after wounding. The effects of the bacteria decreased with increasing time between wounding and inoculation. In dark-grown disks the first effect of the bacteria was in inhibiting growth and this was greatest when disks were inoculated immediately after wounding. When the bacteria were applied 24 hours after wounding, this inhibition was overcome and growth was stimulated. The maximum stimulus to growth occurred after 60 hours, but it decreased with further increases in time between wounding and inoculation. The main action of the bacteria in both light and darkness was associated with freshly wounded tissues and was most effective within 12 hours of wounding. This differs from A. tumefaciens which is effective in inducing the formation of large galls when bacteria are applied up to 60 hours after wounding. The association of E. coli-induced proliferation with freshly wounded tissues also differs from that reported by Fallot (1964) for vine tissues inoculated with B. megaterium.

In this case bacteria were able to induce proliferation when applied up to 30 days after the tissues were wounded. With E. coli it is possible that only the contents of freshly wounded cells areable to react with the bacteria with formation of substances inducing cellular proliferation. When the bacteria induce proliferation, the enlarged cells formed at the lower surface of hypocotyl disks collapse, thereby providing more wound substances for the continued production of the stimulating substances by the bacteria.

The reaction of E. coli with freshly wounded tissue was also demonstrated in older decapitated sunflower plants. In these the greatest callus formation occurred when the bacteria were introduced into the tissues at the same time as an IAA - lanolin paste was applied to the cut stem surface. When the paste was applied four days after the stems had been inoculated with E. coli no such stimulation occurred, and in fact the calluses formed were in many cases smaller than those of uninfected plants. The combined action of E. coli and IAA was therefore effective only in freshly wounded tissues.

Experiments using sunflower pith showed that this tissue reacted to inoculation with E. coli quite differently from disks of hypocotyl. On a complex medium containing both kinetin and IAA the bacteria markedly inhibited growth, while on a simple sucrose-inorganic salts medium the bacteria stimulated growth slightly. In comparison, pith tissue inoculated with A. tumefaciens was inhibited to a smaller extent on the complex medium while

growth was greatly stimulated on the simple medium. Again E. coli showed quite a different action on the tissues from that of A. tumefaciens. Histological studies showed that E. coli had little effect on pith tissue of the hypocotyl disks, so that these results with inoculated pith tissue were not unexpected.

Jablonski and Skoog (1954) have shown that the cambium of tobacco provides a cell division factor for pith tissue so that normal growth occurs when such tissues are supplied with auxin. When tobacco pith alone is grown on medium containing auxin the cells enlarge but do not divide. Sunflower pith tissues may be similar to those of tobacco in requiring cell division factors from adjacent vascular tissues for normal growth. If this were so it might be supposed that the complex growth factors in Skoog's medium supplied the necessary stimulants to cell division and expansion of sunflower pith tissue, but these were not effective in the presence of E. coli. In addition, the bacteria were unable to replace these factors when the pith tissue was grown on the simple medium.

The nature of the stimulus to growth caused by E. coli is clearly different from that of A. tumefaciens or B. megaterium. With A. tumefaciens it has been postulated that a tumour-inducing principle produced by the bacteria in contact with freshly wounded tissues is responsible for the change from normal to tumour cells but the exact nature of this stimulus is not known. Although A. tumefaciens produces IAA in culture this has been eliminated as a possible tumour-inducing principle. Evidence

points to the fact that the tumour-inducing principle is a substance which acts on a basic cellular process and in this way is able to change the pattern of growth permanently.

The nature of the stimulus produced by B. megaterium has been determined. The culture filtrate from which the bacteria had been eliminated was shown to induce proliferation of the same kind as that caused by direct contact between the bacteria and plant tissues. Fallot (1964) analysed this filtrate and characterized several active fractions. He showed that the active indole compounds were of a different nature from any known auxins, because of the nature of the proliferation they induced in certain vine tissues. Although IAA was also found in the filtrate it was not active by itself in inducing the proliferation. No compounds with gibberillin-like activity were found, but two fractions showing affinities with cytokinins were isolated. It was evident from this work that B. megaterium, a non-pathogenic bacterium, produces in its culture medium a wide range of substances active in cell division and expansion, and in the stimulation of root formation.

Corynebacterium fascians has not been considered in this work since it induces organized proliferation, but it is of note that cytokinins have been isolated from culture filtrates of this bacterium (Klambt, Thies and Skoog 1966).

Although B. megaterium can only stimulate proliferation in certain tissues and is not active in sunflower, it is likely that E. coli can similarly produce a wide range of substances in culture, some of which are active in stimulating cell division and expansion.

However, these substances would appear to be active only in conjunction with freshly-wounded tissues and to be dependent on the growth conditions of the sunflower tissues, particularly after inoculation. Although the nature of the active substances appeared from general observations of light-grown disks to be similar to IAA, the tissue proliferation on closer observation differed from that induced by 0.01 ppm IAA. It is known that E. coli produces IAA from tryptophane, but it is apparent that the active substance is not IAA alone, but may be a combination of indole compounds and purine derivatives as in B. megaterium. Burkholder and McVeigh (1942) found that certain vitamins were produced in the culture medium of E. coli, and these may influence the growth-stimulating properties of the bacteria. Vitamins are included in a number of media to promote growth in plant tissue cultures (Gautheret 1959), and the vitamins produced by E. coli may act in a similar manner.

The difference in response of light and dark-grown tissues to inoculation with E. coli may be due entirely to the different growth patterns of the tissues in these conditions. Kuraishi and Muir (1964) showed that sunflowers produce different auxins in the light and the dark, and the bacterial stimulus to growth may react in different ways with these substances. For disks grown in the light the bacterial stimulus to growth continues for some time after inoculation. Initially, wounded tissues are required and the contents of these in some way combine with the bacteria or their products to stimulate growth. The enlarged

cells which are formed as a result of the stimulus rupture so that more products of wounded cells may be made available for continued cell enlargement in the disks.

The action of E. coli on the sunflower tissue, while not being correlated closely with either A. tumefaciens or B. megaterium, is similar to many of the agents of abnormal growth discussed in Chapter 1. In practically all cases wounded tissue is a prerequisite before abnormal growth can be induced by the particular agent. Although the widely differing agents produce growth forms which are morphologically similar, they are in most cases histologically and physiologically different. With all these forms of abnormal growth, the growth substance metabolism seems to be affected and this appears to be true also of hypocotyl disks inoculated with E. coli since the cells are stimulated to divide and expand. However, the exact nature of the growth induced by E. coli cannot be equated with any of the known agents causing abnormal growth. It is closest to growth induced by auxins and is probably caused by a combination of growth factors including indole compounds and purine derivatives.

Further lines of investigation in characterizing the nature of the bacterial stimulus to growth are at once apparent. It should be determined whether contact between the bacteria and plant tissues is necessary before stimulation can occur. If this contact is found unnecessary a characterization of the active compounds present in the culture filtrate would provide the answer to the problem. Because of the association of wounding with stimulation

of growth by bacteria, the action may however be more complex Growth of bacteria in extracts of sunflower tissue may result in the production of the particular substances which cause the characteristic stimulation of sunflower hypocotyl tissues.

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APPENDIX I

TABLES AND CALCULATIONS FOR CHAPTER THREE

EXPERIMENT 1

A comparison of dark-grown decapitated hypocotyls inoculated with *E. coli* and sterile nutrient broth.

TREATMENT	CALLUSING	NO REACTION OR DEATH	TOTAL
E. coli.	9	8	17
Nutrient broth	7	9	16
Total	16	17	33

$$\chi^2 = \frac{(9 \times 9 - 8 \times 7)^2 \, 33}{16 \times 17 \times 16 \times 17}$$

$$= 0.0322 \text{ NS.}$$

A comparison of dark-grown decapitated hypocotyls inoculated with *E. coli* and sterile distilled water.

TREATMENT	DEATH	NO REACTION OR CALLUSING	TOTAL
E. coli	1	16	17
Distilled water	8	9	17
Total	9	25	34

$$\chi^2 = \frac{(9 - 8 \times 16)^2 \, 34}{9 \times 25 \times 17 \times 17}$$

$$= 7.404.$$

Using a correction for continuity (Steel and Torrie 1960)

$$\text{Adjusted } \chi^2 = \frac{(|9 - 8 \times 16| - 34/2)^2 \, 34}{9 \times 25 \times 17 \times 17}$$

$$= 5.44^* \text{ at 2.5\% level.}$$

EXPERIMENT 2

A comparison of dark-grown decapitated hypocotyls inoculated with the original strain of *E. coli* and ECS_4.

TREATMENT	CALLUSES +++ AND LARGER	SMALLER CALLUSES NO REACTION OR DEATH	TOTAL
ECS_4	12	29	41
E. coli	9	11	20
Total	21	40	61

$$\chi^2 = \frac{(12 \times 11 - 9 \times 29)^2 \, 61}{21 \times 40 \times 41 \times 20}$$

$$= 1.47 \text{ NS.}$$

A comparison of dark-grown decapitated hypocotyls inoculated with ECS_4 and sterile nutrient broth.

TREATMENT	CALLUSES +++ AND LARGER	SMALLER CALLUSES NO REACTION OR DEATH	TOTAL
ECS_4	12	29	41
Nutrient broth	3	18	21
Total	15	47	62

$$\chi^2 = \frac{(12 \times 18 - 3 \times 29)^2 \, 62}{15 \times 47 \times 41 \times 21}$$

$$= 1.70 \text{ NS.}$$

A comparison of dark-grown decapitated hypocotyls inoculated with *E. coli* and sterile nutrient broth.

TREATMENT	CALLUSES +++ AND LARGER	SMALLER CALLUSES NO REACTION OR DEATH	TOTAL
E. coli	9	11	20
Nutrient broth	3	18	21
Total	12	29	41

$$\chi^2 = \frac{(9 \times 18 - 3 \times 11)^2 \, 41}{12 \times 29 \times 20 \times 21}$$

$$= 4.67$$

$$\text{Adjusted } \chi^2 = \frac{(|9 \times 18 - 3 \times 11| - 41/2)^2 \, 41}{12 \times 29 \times 20 \times 21}$$

$$= 3.30^* \text{ at 10\% level.}$$

Calculations for the number of replicates required.

The number of replicates of the bacterial and uninfected treatments required to show a significant difference at the 1% level, in the number of large galls formed, 75% of the time, was calculated from the formula of Paulson and Wallis as given in Steel and Torrie (1960).

$$n = 1641.6 \frac{Z_\alpha + Z_\beta}{\arcsin \sqrt{p_S} - \arcsin \sqrt{p_E}}$$

where n was the number of observations in each sample, Z_α the normal deviate such that $p(Z \geqslant Z_\alpha) = \alpha$ and Z_β, $p(Z \geqslant Z_\beta) = \beta$. These were tabulated in Steel and Torrie. p_S was the proportion associated with the nutrient broth treatment, and p_E that for the E. coli treatment.

TREATMENT	CALLUSES (++++)	TOTAL HYPOCOTYLS
E. coli	3	20
Nutrient broth	0	21

For a probability of 99% = 0.01
Estimate of p_S = 0/21
Estimate of p_E = 3/20

Substituting in the equation:-

n, the number of replicates = $1641.6 \left(\frac{3.327 + 0.675}{22.79}\right)^2$

which simplifies to n = 29.

EXPERIMENT 4

TABLE 1. FRESH AND DRY WEIGHTS OF 20 HYPOCOTYL DISKS (g)

AGE	BACTERIAL TREATMENT	LIGHT TREATMENT OF DISKS					
		High Light		Low Light		Dark	
		F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
7 days	Nutrient broth	0.3240	0.0239	0.3835	0.0231	0.2882	0.0204
	E. coli	0.4090	0.0263	0.1519	0.0126	0.0960	0.0086
		0.2775	0.0181	0.1131	0.0068	0.1358	0.0114
						0.1810	0.0150
	A. tumefaciens	0.4549	0.0260	0.5464	0.0250	0.5669	0.0276
14 days	Nutrient broth	0.6123	0.0445	0.8765	0.0496	0.5544	0.0398
	E. coli	1.0006	0.0641	0.1215	0.0108	0.1308	0.0121
		1.1203	0.0802	0.1516	0.0128	0.1863	0.0136
		1.4667	0.0910			0.1255	0.0130
						0.2298	0.0215
						0.1940	0.0202
	A. tumefaciens	0.8736	0.0496	0.5950	0.0311	0.7270	0.0455
22*days	Nutrient broth	0.3500	0.0272	0.9145	0.0658	1.2809	0.0796
21 days	*E. coli*			0.1390	0.0138	0.2025	0.0191
				0.1244	0.0140	0.2531	0.0161

* Taken as 21 days for the purposes of Figure 3.3.
The variable numbers in several treatments were due to contamination.

TABLE 2. AVERAGE DRY WEIGHT/AVERAGE FRESH WEIGHT

AGE	BACTERIAL TREATMENT	LIGHT TREATMENT OF DISKS		
		High Light	Low Light	Dark
7 days	Nutrient broth	0.0738	0.0602	0.0708
	E. coli	0.0647	0.0732	0.0863
	A. tumefaciens	0.0572	0.0458	0.0487
14 days	Nutrient broth	0.0727	0.0566	0.0718
	E. coli	0.0638	0.0864	0.0929
	A. tumefaciens	0.0568	0.0539	0.0626
22*days	Nutrient broth	0.0777	0.0720	0.0621
21 days	E. coli		0.1055	0.0773

* Taken as 21 days for the purpose of Figure 3.4

EXPERIMENT 5

TABLE 3. FRESH AND DRY WEIGHTS OF 10 HYPOCOTYL DISKS (g)

AGE	SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT					
			High Light		Low Light		Dark	
			F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
7 days	High light	Nutrient broth	0.1630	0.0130	0.1248	0.0088	0.1373	0.0089
			0.1537	0.0119	0.1155	0.0080	0.1232	0.0076
	Low light		0.2163	0.0145	0.1250	0.0073	0.2335	0.0155
			0.1420	0.0110	0.1174	0.0074	0.2347	0.0133
	Dark		0.1278	0.0085	0.0832	0.0056	0.2575	0.0170
			0.1805	0.0121	0.2122	0.0115	0.2011	0.0125
	High Light	*E. coli*	0.2315	0.0149	0.1170	0.0086	0.1347	0.0108
			0.2635	0.0170	0.0892	0.0073	0.0837	0.0069
			0.2209	0.0235	0.1680	0.0116	0.0659	0.0045
			0.2480	0.0177	0.0963	0.0079	0.0713	0.0058
	Low Light		0.1334	0.0104	0.1393	0.0094	0.0975	0.0070
			0.1896	0.0117	0.1295	0.0089	0.0670	0.0050
			0.1841	0.0117	0.0997	0.0068	0.0886	0.0057
			0.1494	0.0090	0.1084	0.0075	0.0740	0.0050
	Dark		0.2051	0.0126	0.1656	0.0104	0.2200	0.0151
			0.1521	0.0100	0.1117	0.0077	0.0675	0.0060
			0.1402	0.0087	0.1264	0.0086	0.0898	0.0059
			0.1339	0.0103	0.0788	0.0055	0.0818	0.0060
	High Light	*A. tumefaciens*	0.1936	0.0116	0.1845	0.0100	0.1416	0.0102
			0.1836	0.0122	0.2064	0.0123	0.1212	0.0090
	Low Light		0.2521	0.0140	0.1345	0.0088	0.1428	0.0099
			0.2100	0.0115	0.1529	0.0104	0.1657	0.0119
	Dark		0.2594	0.0133	0.1202	0.0065	0.1945	0.0120
			0.2200	0.0121	0.0988	0.0066	0.2132	0.0104
14 days	High Light	Nutrient broth	0.1753	0.0141	0.1847	0.0117	0.3074	0.0186
			0.1497	0.0120	0.2830	0.0181	0.1953	0.0125
	Low Light		0.4332	0.0312	0.2070	0.0139	0.4470	0.0296
			0.5841	0.0345	0.2358	0.0143	0.4553	0.0304
	Dark		0.4872	0.0330	0.2830	0.0185	0.5485	0.0405
			0.7016	0.0417	0.2863	0.0182	0.4767	0.0315
	High Light	*E. coli*	0.4935	0.0362	0.1775	0.0140	0.0860	0.0073
			0.2136	0.0213	0.1695	0.0129	0.0920	0.0080
			0.2008	0.0190	0.1595	0.0122	0.1009	0.0097
			0.2567	0.0210	0.1600	0.0116	0.0653	0.0073
	Low Light		0.3370	0.0254	0.1615	0.0127	0.0856	0.0067
			0.7212	0.0442	0.1900	0.0135	0.1138	0.0163
			0.2614	0.0217	0.2422	0.0160	0.0972	0.0069
			0.7001	0.0442	0.1396	0.0110	0.0652	0.0049

TABLE 3 Cont'd.

AGE	SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT					
			High Light		Low Light		Dark	
			F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
14 days	Dark	E. coli	1.8266	0.1120	0.1323	0.0092	0.0504	0.0038
			0.1409	0.0120	0.1335	0.0113	0.0880	0.0085
			0.1396	0.0120	0.1087	0.0082	0.0856	0.0080
			0.1356	0.0123	0.3092	0.0197	0.1127	0.0093
			0.1410	0.0104				
	High Light	A. tume-faciens	0.2742	0.0170	0.3513	0.0160	0.2276	0.0130
			0.2100	0.0140	0.3135	0.0160	0.2412	0.0137
	Low Light		0.3121	0.0165	0.3020	0.0160	0.2067	0.0125
			0.4291	0.0255	0.3857	0.0208	0.1673	0.0108
	Dark		0.2978	0.0179	0.2610	0.0134	0.2145	0.0124
			0.3291	0.0212	0.2899	0.0141	0.2546	0.0149
21 days	High Light	Nutrient broth	0.6274	0.0380	0.3490	0.0210	0.5840	0.0380
			0.1756	0.0138	0.5290	0.0350	0.4484	0.0278
	Low Light		0.2115	0.0140	0.5367	0.0325	1.1040	0.0810
			0.3345	0.0258	0.3175	0.0200	0.3556	0.0217
	Dark		0.8178	0.0530	0.3874	0.0276	0.8339	0.0585
			0.4801	0.0325	0.5714	0.0358	1.0947	0.0780
	High Light	E. coli	1.0377	0.0698	0.1915	0.0166	1.2907	0.0881
			0.1996	0.0177	0.1669	0.0164	1.1366	0.0748
			0.2784	0.0257	0.1014	0.0085	0.2051	0.0146
			0.4485	0.0365	0.1900	0.0156	0.0413	0.0030
	Low Light		0.5359	0.0374	0.2009	0.0168	0.0826	0.0052
			1.2320	0.0715	0.2019	0.0154	0.0728	0.0044
			0.2860	0.0245	0.1798	0.0130	0.1213	0.0095
			0.2669	0.0225	0.3337	0.0242	0.0631	0.0065
	Dark		0.0399	0.0035	0.2678	0.0193	0.0965	0.0077
			0.1676	0.0161	0.1002	0.0074	0.0758	0.0070
			1.1266	0.0720	0.1708	0.0096	0.0606	0.0050
			2.2927	0.1457	0.1421	0.0117	0.0686	0.0052
	High Light	A. tume-faciens	0.3456	0.0220	0.3498	0.0196	0.1790	0.0134
			0.4148	0.0238			0.2979	0.0187
	Low Light		0.3860	0.0250	0.3703	0.0208	0.1542	0.0102
			0.3875	0.0243	0.4135	0.0235	0.2673	0.0153
	Dark		0.3705	0.0242	0.2692	0.0143		
			0.5288	0.0327	0.4005	0.0229		
28 days	High Light	Nutrient broth	0.3871	0.0307	0.3328	0.0223	0.3081	0.0291
			0.3442	0.0285	0.3446	0.0210	0.8146	0.0654
	Low Light		0.2067	0.0155	0.7558	0.0462	1.2821	0.0964
			0.3563	0.0290	0.3640	0.0230	0.7753	0.0530

TABLE 3 Cont'd.

AGE	SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT					
			High Light		Low Light		Dark	
			F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
28 days	Dark	Nutrient broth	3.1884	0.1970	0.4890	0.0315	1.5008	0.1047
					2.8131	0.1796	0.8103	0.0580
	High Light	*E. coli*	0.1993	0.0218	0.2354	0.0185	0.1848	0.0165
			0.3188	0.0292	0.1750	0.0150	0.1469	0.0101
			0.4002	0.0400	0.1079	0.0094	0.2484	0.0205
			3.3786	0.1848			0.9320	0.0636
	Low Light		1.4206	0.0858	0.5477	0.0364	0.0532	0.0036
			4.1375	0.2270	0.0942	0.0061	0.0870	0.0084
			2.3604	0.1404	0.2333	0.0196	0.0865	0.0055
			1.5584	0.1089	0.6361	0.0472	1.5718	0.1087
	Dark		2.0843	0.1448	0.1125	0.0060	0.1304	0.0114
					0.1226	0.0095	0.1107	0.0100
					0.0998	0.0068	0.2845	0.0291
					0.1775	0.0125	0.1041	0.0072
							0.0984	0.0073
							0.0973	0.0067
							0.1251	0.0111
							0.1705	0.0160
	Dark	*A. tume-faciens*					0.4268	0.0340
							0.3467	0.0260

TABLE 4. GROWTH RESPONSES OF HYPOCOTYL DISKS RECORDED BY MEANS OF SYMBOLS

PA - Proliferation from the upper surface.
PB - Proliferation from the lower surface.
PE - Proliferation from the lower surface so that the epidermal tissues are pushed out.
D - Disks dead.
RN - No roots.
RS - Roots up to 1 cm long.
RM - Roots 1-4 cm long.
RL - Roots longer than 4 cm.

(The numbers represent the number of disks in one hypocotyl showing the particular reaction.)

AGE	SEEDLING-LIGHT TREATMENT	BACTERIAL TREATMENT	DISK-LIGHT TREATMENT		
			High Light	Low Light	Dark
7 days	High Light	Nutrient broth	PBRN	PARN	PAPBRS
			PBRN	PARN	PAPBRN
	Low Light		PBPERN	PARN	PAPBRS
			PBPERN	PARN	PAPBRL
	Dark		PAPB2RS	PARN	PAPBRS
			PAPBRN	PA1RS	PAPBRM
	High Light	E. coli	PBPERN	8PN2DRN	D2RM
			PBPERN	PNRN	6PN4DRN
			8PBPE2D	7PN3PARN	DRN
			PBPERN	PNRN	DRN
	Low Light		8PBPE2DRN	PNRN	5PN5DRN
			4PN3PBPERN	PN1RS	4D6PNRN
			PBPERN	PNRN	3PN7DRN
			4PBPE6DRN	PNRN	3PN7DRN
	Dark		6PBPERM4D	PNRN	8PAPBRL2D
			6PBPERS4D	3PA7DRN	4PAPB6DRN
			5PBPE5DRN	7PA2RS3D	4PAPBRS6D
			4PBPE6DRN	4PA1RS6D	4PAPBRS6D
	High Light	A. tume-faciens	8PA2DRN	PARN	8PA2DRN
			PARN	PARN	7PA3DRN
	Low Light		PARN	PARN	6D4PARM
			PARN	PARN	PA1RS
	Dark		PARN	7PA3DRN	PA3RM
			PARN	2PA8DRN	PARN

TABLE 4 Cont'd.

AGE	SEEDLING-LIGHT TREATMENT	BACTERIAL TREATMENT	DISK-LIGHT TREATMENT		
			High Light	Low Light	Dark
14 days	High Light	Nutrient broth	PARN PARN	PA1RS PA3RS	PA6RL PA1RS
	Low Light		7PN3PARN 9PAPBRL1D	PARN PA5RL	PNRL 2PA8PNRL
	Dark		5PA5PBRN PA3RL	7PNRM3D PAPB1RS	3PA7PNRL PARL
	High Light	E. coli	PAPBRL PBRN PBPE2RL PBPERN	8PBPERN2D PAPBRN 9PBPE1D2RS PN1RS	6PN4D1RS 6PN4D2RS 5PN5D1RS 6PN4DRN
	Low Light		PBPE2RL 9PBPE4RL PBPERN1D PBPE7RL	9PA7RM1D 9PA1D7RS PAPBPE4RS 3D7PNRN	DRN PNRN DPNRN DPNRN
	Dark		2PBPE8DRN PBPERL 2PBPE8DRN 4PB6DRN 5PBPE5DRN	6PN4D3RS 7PN3DRN 5PNRS5D 9PBPERL1D	2PA8DRN 9PN1DRN 7PN3DRN 3D7PB5RS
	High Light	A. tumefaciens	9PA1DRN 9PA1DRN	PARN PARN	5PA1RS5D 4D6PARS
	Low Light		PARN PA2RS	PARN PARN	3D7PARM 3PA7D2RS
	Dark		PARN PARN	PA3DRN 2D8PARN	9PA1DRN 9PA1DRN
21 days	High Light	Nutrient broth	PAPB1RL PAPBRN	PA2RL PA4RL	PA5RL PA2RL
	Low Light		PBRN PBRN	PAPB8RL PA1RL	4PA6PNRL PAPBRM
	Dark		PAPB1RL PAPBRN	8PA2RL2D PARL	2PA8PBRL 6PA4PBRL

TABLE 4 Cont'd.

AGE	SEEDLING-LIGHT TREATMENT	BACTERIAL TREATMENT	DISK-LIGHT TREATMENT		
			High Light	Low Light	Dark
21 days	High Light	E. coli	9PBPE6RL1D 5PBPE5DRN PBPERN 9PBPE1DRN	3D7PBPE3RS 8PBPE2DRS 8PN2DRN 9PBPE3RS1D	PAPBPERL 9PBPERL1D 3PN7DRS DRN
	Low Light		9PBPE3RL1D 7PBPE3RL3D 8PBPE1RL2D 9PBPE1RL1D	PBPE1RS 6PBPERS4D 5PBPERS5D 8PBPERS2D	1PNRS9D DRS 6PARS4D 3PNRN7D
	Dark		DRN 4PBPE6DRN 6PBPERL4D 3PBPERL7D	9PBERM1D 3PNRS7D 2PBPERS8D 5PNRS5D	4PNRS6D 4PN6DRN 3PN7DRN 3PNRS7D
	High Light	A. tume-faciens	PARS PARS	8PA2DRN	1PARS9D 3PARS7D
	Low Light		4PA6DRN 7PA3DRN	4PARS6D 6PARS4D	PA2RS 6PA4DRS
	Dark		PARN PARN	PARN PARS	
28 days	High Light	Nutrient broth	8PN2PBRN 8PN2PBRN	PAPBRN PAPB2RL	PAPB2RS PA6RL
	Low Light		PBRN PBRN	PAPB8RL PA3RL	PAPBRL PAPBRL
	Dark		PAPBRL	PN6RL PAPBRL	PAPB2RL PAPB8RL
	High Light	E. coli	9PBPERN1D PBPERN PBPERN 9PBPERL1D	PBPE1DRN 3D7PE4RS 5PE5DRN	6PNRM4D 7DRS3D 5PNRS5D 9PARL1D
	Low Light		4PEPB6DRL 9PBPERL1D 8D2PBPERL	9PBPERL1D 1PBPE9D2RS 7PBPERS3D 9PBPERL1D	DRN D1RS D1RS 8PBPERL2D

TABLE 4 Cont'd.

AGE	SEEDLING-LIGHT TREATMENT	BACTERIAL TREATMENT	DISK-LIGHT TREATMENT		
			High Light	Low Light	Dark
28 days	Dark	E. coli	7PBPERL3D 4PBPERL6D	DRS 4PNRS6D 9D1RS DPN3RS	4PBRS6D 2PERS8D 4PNRS6D 3PN7D1RS 8PBPE2D 4PNRS6D 3PNRS7D 3PBRL7D
	Dark	A. tumefaciens			7PARN3D

TABLE 5. AVERAGE FRESH AND DRY WEIGHTS OF 10 HYPOCOTYL DISKS (g)

AGE	SEED LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT					
			High Light		Low Light		Dark	
			F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
1 week	High Light		0.1584	0.0125	0.1202	0.0084	0.1303	0.0083
	Low Light	Nutrient broth	0.1792	0.0128	0.1212	0.0074	0.2341	0.0144
	Dark		0.1542	0.0103	0.1477	0.0086	0.2293	0.0148
	High Light		0.2409	0.0183	0.1176	0.0089	0.0889	0.0070
	Low Light	*E. coli*	0.1641	0.0107	0.1192	0.0082	0.0818	0.0057
	Dark		0.1578	0.0102	0.1206	0.0081	0.1148	0.0083
	High Light		0.1886	0.0119	0.1955	0.0112	0.1314	0.0096
	Low Light	*A. tumefaciens*	0.2311	0.0128	0.1437	0.0096	0.1543	0.0109
	Dark		0.2397	0.0127	0.1095	0.0066	0.2039	0.0112
2 weeks	High Light		0.1625	0.0131	0.2339	0.0149	0.2514	0.0156
	Low Light	Nutrient broth	0.5087	0.0329	0.2214	0.0141	0.4512	0.0300
	Dark		0.5944	0.0374	0.2847	0.0184	0.5126	0.0360
	High Light		0.2912	0.0244	0.1666	0.0127	0.0861	0.0081
	Low Light	*E. coli*	0.5049	0.0339	0.1833	0.0133	0.0905	0.0072
	Dark		0.4767	0.0317	0.1709	0.0121	0.0842	0.0074
	High Light		0.2421	0.0155	0.3324	0.0160	0.2344	0.0134
	Low Light	*A. tumefaciens*	0.3706	0.0210	0.3439	0.0184	0.1870	0.0117
	Dark		0.3135	0.0196	0.2755	0.0138	0.2346	0.0137
3 weeks	High Light		0.4015	0.0259	0.4390	0.0280	0.5162	0.0329
	Low Light	Nutrient broth	0.2730	0.0199	0.4271	0.0263	0.7298	0.0514
	Dark		0.6489	0.0428	0.4794	0.0317	0.9643	0.0683
	High Light		0.4911	0.0374	0.1625	0.0143	0.6684	0.0451
	Low Light	*E. coli*	0.5802	0.0389	0.2291	0.0174	0.0849	0.0064
	Dark		0.9067	0.0593	0.1702	0.0120	0.0754	0.0062
	High Light		0.3802	0.0229	0.3498	0.0196	0.2385	0.0161
	Low Light	*A. tumefaciens*	0.3868	0.0247	0.3919	0.0222	0.2108	0.0128
	Dark		0.4497	0.0285	0.3349	0.0186		

TABLE 5 Cont'd.

AGE	SEED LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT					
			High Light		Low Light		Dark	
			F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
4 weeks	High Light	Nutrient broth	0.3657	0.0296	0.3387	0.0217	0.5614	0.0473
	Low Light		0.2815	0.0223	0.5599	0.0346	1.0287	0.0747
	Dark		3.1884	0.1970	1.6511	0.1056	1.1556	0.0814
	High Light	*E. coli*	1.0742	0.0689	0.1761	0.0143	0.3780	0.0277
	Low Light		2.3692	0.1405	0.3778	0.0273	0.4496	0.0316
	Dark		2.0843	0.1448	0.1281	0.0087	0.1401	0.0124
	Dark	*A. tumefaciens*					0.3868	0.0300

EXPERIMENT 6

A comparison of dark-grown decapitated hypocotyls inoculated with a suspension of *E. coli* in distilled water and nutrient broth.

TREATMENT	CALLUSING	NO REACTION OR DEATH	TOTAL
E. coli suspension	12	10	22
E. coli in broth	7	9	16
	19	19	38

$$\chi^2 = \frac{(12 \times 9 - 7 \times 10)^2 \, 38}{19^2 \times 22 \times 16}$$

$$= 0.493 \text{ NS.}$$

APPENDIX II

CALCULATION FOR CHAPTER FOUR

EXPERIMENT 7c

A comparison of the numbers of roots produced in the hypocotyls of E. coli-inoculated and uninfected intact sunflowers.

From Table 4.2, frequency table.

$$\begin{vmatrix} 5 & 5 & 0 \\ 2 & 1 & 7 \end{vmatrix} \qquad \begin{vmatrix} 3.5 & 3 & 3.5 \\ 3.5 & 3 & 3.5 \end{vmatrix}$$

$$\chi^2 = 2\left(\frac{1.5^2}{3.5} + \frac{4}{3} + \frac{3.5^2}{3.5}\right)$$

$$= 10.94 \text{ with } 2 \text{ D.F.* at } 1\%$$

APPENDIX III

TABLES AND CALCULATIONS FOR CHAPTER FIVE

EXPERIMENT 9

TABLE 1. GROWTH RESPONSES OF HYPOCOTYL DISKS RECORDED BY MEANS OF SYMBOLS

Each treatment shown represents the general reaction of ten replicates.

PA - Proliferation from the upper surface.
PB - Proliferation from the lower surface.
PE - Proliferation from the lower surface so that the epidermal tissues are pushed out.
D - Disks dead

RN - No roots
RS - Roots up to 1 cm long
RM - Roots 1-4 cm long
RL - Roots longer than 4 cm.

IAA conc (ppm)	BACTERIAL TREATMENT	SEEDLINGS AND DISKS GROWN IN THE LIGHT							
		THREE WEEKS		FOUR WEEKS		FIVE WEEKS		SIX WEEKS	
		GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK
0	Uninfected	PAPB4RL	1-2	PAPB6RL	1-2	PAPB5RL	1	PAPB5RL	1-2
	E. coli	PBPERL	2-3	PBPERL	1	PBPERL	2	PBPERL	1-2
	A. tumefaciens	PAPBRS	2-3	PARNorS	0-6	PAPBRS	5-6	PARNorS	0-6
0.01	Uninfected	PAPBPERL	2-3	PAPBPERL	1-2	PAPBRL	1-5	PAPBPERL	1-2
	E. coli	PBPERL	5	PAPBPERL	2-5	PBPERL	3-4	PBPERL	1-5
	A. tumefaciens	PARM	5-6	PAPBRM	1-5	PAPBRS	6	PARS	1-6
0.1	Uninfected	PBPERSorL	2-6	PBPERL	1-2	PBPERL	1-5	PBPERL	1-4
	E. coli	PBPERL	6	PBPERL5D	1-6	PBPERL	1-6	PBPERM	5-6
	A. tumefaciens	PAPERSorM	5-6	PARS9D	1-5	PAPERM	1-6	PAPERM	1-5
1.0	Uninfected	PAPBPERS	5-6	PAPERS	5	PAPE6RS	1-6	PBPERSorL	1-5
	E. coli	PBRNorS	0-5	DRNorS	0	DPARNorS	0-3	DRMNorS	0-5
	A. tumefaciens	PAPERN	0	PAPERN	0	PARM	0-6	DPA3RM	2-3
10	Uninfected	PARNorS	1-6	PAPERSorN	1-6	PAPERNorS	1-6	PARNorS	1-6
	E. coli	DRNorS	0-4	DRN	0	DRN	0	DRNorS	0-2
	A. tumefaciens	DPARN	0	DPARN	0	DPARN	0	DPARN	0

TABLE 1 CONT'D.

SEEDLINGS GROWN IN THE DARK, DISKS IN THE LIGHT

IAA conc. (ppm)	BACTERIAL TREATMENT	THREE WEEKS GROWTH	THREE WEEKS ROOT NO. PER DISK	FOUR WEEKS GROWTH	FOUR WEEKS ROOT NO. PER DISK	FIVE WEEKS GROWTH	FIVE WEEKS ROOT NO. PER DISK	SIX WEEKS GROWTH	SIX WEEKS ROOT NO. PER DISK
0	Uninfected	PAPBRL	1-2	PAPBRL	2-3	PAPBRL	2	PAPBRL	2-3
	E. coli	PBPERL	1-2	PBPERL	2-3	PBPERL	3-4	PBPERL	3-4
	A. tumefaciens	PARS	1-5	PARS	1-6	PARS	4-5	PARS	5-6
0.01	Uninfected	PAPBPERL	2-3	PAPBRL	3-4	PAPBRL	1-6	PAPBRL	5-6
	E. coli	PBPERL	1-6	PBPERL	3-4	PBPERL	5-6	PBPERL	1-2
	A. tumefaciens	PARM	2-3	PAPBRL	4-5	PAPBRL	1-6	PAPBRL	3-4
0.1	Uninfected	PBPERSorL	3-6	PAPBPERL	1-6	PBPERL	1-5	PBPERL	5-6
	E. coli	PBPERLD	5	PBPERLD	4-5	PAPBPERLD	6	PBPERSD	4-5
	A. tumefaciens	PAPERM	3	PARSNorL	4-5	PARL	5-6	PARSNorL	5-6
1.0	Uninfected	PAPBPERS	5-6	PAPBPERS	1-6	PAPBPERS	1-6	PAPERS	6-7
	E. coli	DPBRNorS	3-4	DRS	3-4	DRNorS	1-4	PBPERLD	3-4
	A. tumefaciens	PAPBRM	1-3	PAPBRSLorM	1-6	DPARN	0	PARSorND	5-6
10	Uninfected	PBRS	5-6	PARS	1-6	PAPERS	5-6	PARNorS	5-6
	E. coli	DRNorS	2-3	DRN	0	DRN	0	DRN	0
	A. tumefaciens	PAPERN	0	DPARN	0	DPARN	0	DPARN	0

SEEDLINGS GROWN IN THE LIGHT, DISKS IN THE DARK

0	Uninfected	PAPBRL	2-3	PAPBRL	1-5	PAPBRL	2-3	PBRL	5-6
	E. coli	PARSorND	0-3	PBPERSD	5-6	PBPERSD	5	PARSorND	0-3
	A. tumefaciens	PARSD	4-5	PARS	2-4	PARS	5-6	PARS	4-5
0.01	Uninfected	PAPBPERL	5-6	PAPBPERL	4-6	PAPBRL	6	PBPERL	5-6
	E. coli	PAPBRS	3-4	PAPBRLorS	5-6	PBPERSD	5	DPARS	3-4
	A. tumefaciens	PARS	4-5	PARS	5-6	PARSD	4	PARS	5-6
0.1	Uninfected	PAPBRS	4-5	PAPERS	4-5	PAPERS	5-6	PAPERS	4-5
	E. coli	DPARS	4-5	DRNorS	0-4	DPERNorS	0-3	DRNorS	0-3
	A. tumefaciens	DPARL	4-5	PARMorS	4-5	PARMD	5-6	DPARS	4-5

TABLE 1 CONT'D		SEEDLINGS GROWN IN THE LIGHT, DISKS IN THE DARK - CONT'D							
		THREE WEEKS		FOUR WEEKS		FIVE WEEKS		SIX WEEKS	
IAA conc. (ppm)	BACTERIAL TREATMENT	GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK	GROWTH	ROOT NO. PER DISK
1.0	Uninfected	PARNorS	0-6	PARS	5-6	PARS	5-6	PARS	5-6
	E. coli	DRNorS	0-6	DRN	0	DRN	0	DRN	0
	A. tumefaciens	DPARS	4-5	DPARNorS	0-4	DPARN	0	DPARNorS	0-3
10	Uninfected	PARN	0	PARN	0	PARNorS	0-4	PARS	4-5
	E. coli	DRN	0	DRN	0	DRN	0	DRN	0
	A. tumefaciens	DRN	0	DRN	0	DRN	0	DRN	0
SEEDLINGS AND DISKS GROWN IN THE DARK									
0	Uninfected	PAPBRL	5-6	PAPBRL	2-3	PAPBRL	5-6	PAPBRL	4-5
	E. coli	PARS	1-2	PARSD	2-3	DPARS	2-3	DPARSorN	0-3
	A. tumefaciens	PARS	5-6	PAPBRS	4-5	PARNorS	0-3	PARS	4-5
0.01	Uninfected	PAPBRL	5-6	PAPBRL	5-6	PAPBRL	5-6	PARL	4-6
	E. coli	PAPBRL	3-6	PAPBRLorS	5-6	PBRLD	5-6	PARL	5-6
	A. tumefaciens	PAPBRL	0-4	PARS	2-3	PARM	5-6	PARSorL	5-6
0.1	Uninfected	PBPERS	6	PAPBRS	1-6	PAPERS	1-6	PARS	1-6
	E. coli	DRN	0	DRNorS	0-6	DPARNorS	0-3	DPARS	1-3
	A. tumefaciens	DPARN	0	PARS	2-3	PARLD	2-3	PARNorS	0-3
1.0	Uninfected	PAPERS	6	PARSorN	0-6	PARS	1-6	PARNorS	0-6
	E. coli	DRN	0	DRN	0	DPARN	0	DRNorS	2-3
	A. tumefaciens	DPARN	0	PAPBRNorL	1-6	DPARN	0	PARSorL	1-2
10	Uninfected	PARN	0	PARN	0	PARN	0	PARSorN	0-3
	E. coli	DRN	0	DRN	0	DRN	0	DRN	0
	A. tumefaciens	DRN	0	DPARN	0	DPARN	0	DRN	0

TABLE 2. INITIAL FRESH WEIGHTS OF HYPOCOTYLS EACH COMPRISING TEN DISKS ($g \times 10^3$)

SEEDLINGS GROWN IN LIGHT		SEEDLINGS GROWN IN DARK	
DISKS SUBSEQUENTLY GROWN IN:		DISKS SUBSEQUENTLY GROWN IN:	
Light	Dark	Light	Dark
276	295	230	386
304	350	216	356
396	215	317	321
348	300	266	320
291	260	239	327
389	312	286	336
674	345	189	273
317	235	378	277
519	280	296	227
345	276	327	357
Average weight 385.9	286.8	274.4	318.0

ANALYSIS OF VARIANCE FOR INITIAL FRESH WEIGHTS

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F
Between treatments	74,729	3	24,910	4.4 * 1%
Within treatments	203,363	36	5,649	
Total	278,092	39		

TABLE 3. AVERAGE FINAL WEIGHT/AVERAGE INITIAL WEIGHT FOR BOTH FRESH AND DRY WEIGHTS

SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	THREE WEEKS AFTER INOCULATION									
		Light DISK LIGHT TREATMENT Dark									
		Concentration of IAA (ppm)					Concentration of IAA (ppm)				
		0	0.01	0.1	1.0	10	0	0.01	0.1	1.0	10
Fresh weights											
Light	Uninfected	24.4181	71.8145	81.6595	59.9437	22.4251	56.9793	81.5275	89.4137	47.6068	12.2034
	E. coli	75.6756	86.6282	75.6085	12.6549	6.2406	18.2689	20.0310	6.1068	4.7689	3.3379
	A. tumefaciens	20.9310	24.4031	29.2520	27.1867	13.1746	29.1586	31.1965	43.3724	27.2931	4.9551
Dark	Uninfected	73.6507	99.2310	124.6186	91.1580	65.5248	46.9062	59.1781	47.1718	64.3562	8.5406
	E. coli	109.9978	116.0416	73.3445	12.7010	14.7575	7.8062	30.5343	5.5718	4.1343	2.8562
	A. tumefaciens	37.8711	45.6087	43.3040	28.6452	23.0240	26.9125	28.6937	22.6281	19.9031	3.3687
Dry weights											
Light	Uninfected	15.3886	42.8272	47.6227	33.3159	13.4409	26.1500	42.2500	42.9000	21.1000	5.3750
	E. coli	42.9454	51.7204	44.2454	7.2227	3.7590	9.1750	9.9500	2.6500	2.0750	1.6750
	A. tumefaciens	9.9886	13.1090	16.2636	17.4000	8.4136	12.8000	14.6250	20.5000	13.0500	2.3500
Dark	Uninfected	48.5875	72.2166	97.8000	62.1791	44.1791	36.7000	46.6333	33.2666	46.2000	5.2000
	E. coli	82.0625	91.0916	56.1416	5.2666	11.3916	6.7000	24.7000	3.4000	2.4000	1.8000
	A. tumefaciens	28.0750	34.9458	34.1666	23.5041	18.0875	17.2333	19.1666	16.1666	12.9666	2.1000
		FOUR WEEKS AFTER INOCULATION									
Fresh weights											
Light	Uninfected	48.8000	80.2028	98.9204	76.9567	33.0497	91.5310	80.5965	72.8068	68.7655	12.5551
	E. coli	80.4909	91.0823	92.0248	10.2826	8.2531	23.6896	16.5551	8.5448	3.2896	2.3172
	A. tumefaciens	23.1000	38.9409	33.8652	31.8494	15.1388	30.4827	31.5241	47.9620	17.3965	7.5931
Dark	Uninfected	96.0105	109.3948	134.2671	128.1343	70.0259	55.3968	63.5125	67.0468	43.6093	9.5343
	E. coli	130.1412	138.8580	85.4645	25.3364	4.6762	7.8437	34.3187	7.5250	4.1187	2.8031
	A. tumefaciens	33.8076	105.2416	43.4667	44.9777	25.7193	27.4031	31.8125	35.4218	27.2718	3.9906
Dry weights											
Light	Uninfected	27.8772	52.5681	58.6704	41.3613	21.4568	47.5500	41.4000	28.8500	33.2750	6.0750
	E. coli	50.4931	54.6522	52.3022	5.4818	4.5636	11.7500	9.6250	4.3500	1.4750	1.1250
	A. tumefaciens	13.2090	21.7272	21.0772	20.7386	9.6227	14.1750	14.3250	20.7500	8.3500	3.7750

TABLE 3 Cont'd.

FOUR WEEKS AFTER INOCULATION Cont'd.

SEEDLING LIGHT TREATMENT	BACTERIAL TREATMENT	DISK LIGHT TREATMENT: Light — Concentration of IAA (ppm) 0	0.01	0.1	1.0	10	Dark — Concentration of IAA (ppm) 0	0.01	0.1	1.0	10
Dark	Uninfected	75.3625	89.1416	101.6250	95.7125	50.3416	45.3666	54.3666	46.7333	30.7666	6.3333
	E. coli	102.3125	100.4958	67.7791	17.8666	4.1875	6.5333	27.7000	4.8000	2.0000	1.7333
	A.tumefaciens	24.6375	76.3458	35.2291	36.8458	22.2708	19.8666	22.0333	23.4333	18.6000	2.4333
Fresh weights		FIVE WEEKS AFTER INOCULATION									
Light	Uninfected	46.9896	102.0759	112.7388	69.4124	28.1689	98.2172	88.1344	92.1827	63.1172	27.0827
	E. coli	97.1487	115.4974	86.2696	21.5150	5.4264	17.7482	16.1172	13.1379	4.1275	2.7241
	A.tumefaciens	26.1463	30.6150	37.2500	44.4904	16.9518	34.3724	32.1758	45.5965	16.0862	5.5482
Dark	Uninfected	130.4051	147.9554	134.6642	99.6901	102.0536	60.3875	71.1187	53.8375	54.6718	21.0781
	E. coli	127.6740	119.9704	52.2083	22.1478	8.4613	5.7875	30.8812	6.3000	3.5250	2.2875
	A. tumefaciens	42.7927	118.6375	62.2328	29.1313	21.4419	30.0687	28.9406	27.7843	18.8343	4.1875
Dry weights											
Light	Uninfected	30.1772	62.9204	68.0818	38.7863	17.1500	51.2750	49.9000	43.2750	26.4000	12.2000
	E. coli	57.7613	66.0909	54.9340	11.9409	3.3318	9.3250	8.7000	5.5500	1.5500	1.4250
	A.tumefaciens	14.3022	18.4795	21.9363	26.9272	10.4045	17.1250	15.4000	19.5000	7.4750	2.6500
Dark	Uninfected	105.0291	115.3458	103.7541	75.2458	72.8625	52.0666	62.8666	33.6000	39.2333	13.9666
	E. coli	97.7125	95.3916	43.4625	16.5375	6.3916	5.0666	23.3000	4.0333	2.2000	1.4666
	A.tumefaciens	33.9875	87.0666	50.8750	23.7000	18.4875	22.8000	21.6000	20.0666	12.7000	2.8000
Fresh weights		SIX WEEKS AFTER INOCULATION									
Light	Uninfected	71.6463	101.0062	106.9261	79.8670	26.4787	118.2068	94.0931	88.3034	65.3758	32.3620
	E. coli	88.1463	104.0911	97.6020	28.4707	7.3979	27.2758	16.1862	4.3965	4.3034	2.7448
	A.tumefaciens	26.5240	32.6308	39.8917	40.3339	10.0645	38.2172	41.0000	28.9103	27.6413	5.8137
Dark	Uninfected	125.4197	143.1029	149.3905	109.8343	73.0058	73.6656	84.9156	62.1718	53.4312	24.3687
	E. coli	136.2277	120.4394	58.8496	29.8711	3.0861	5.0156	38.8500	4.9812	4.7625	2.7156
	A.tumefaciens	43.1948	121.7423	46.4635	44.8266	27.4102	29.4500	30.5781	19.0093	33.0343	3.6812
Dry weights											
Light	Uninfected	45.4000	65.4863	68.3431	48.0909	17.4681	55.0250	53.7750	40.0000	29.5250	14.1000
	E. coli	57.8045	61.8931	57.7818	17.0613	5.2022	13.2000	9.6250	1.8000	1.5750	1.3000
	A.tumefaciens	15.6136	19.0886	24.3590	26.3272	6.3704	19.9500	21.0000	14.0500	12.7750	3.0000
Dark	Uninfected	103.2750	109.5250	110.6291	81.3166	55.1416	62.0333	71.0666	38.7666	36.0000	16.3000
	E. coli	99.0208	94.3958	46.2583	22.7000	1.9958	4.1666	32.1000	3.5333	2.8333	1.8666
	A.tumefaciens	33.6333	87.0666	36.3666	35.6166	21.8625	24.7666	24.1666	14.1333	24.4666	2.5000

TABLE 4. ANALYSIS OF VARIANCE USING ADJUSTED FRESH WEIGHTS (USING 4 SIGNIFICANT FIGURES)

DISKS THREE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	8,803,638	1	8,803,638	12.67*at 1%
	DL	90,461,849	1	90,461,849	130.24***
	IAA	142,055,890	4	35,513,972	51.13***
	B	133,960,011	2	66,980,005	96.43***
First order Interaction	SLxDL	30,119,752	1	30,119,752	43.36***
	SLxIAA	3,695,710	4	923,927	1.33 NS
	DLxIAA	12,348,710	4	3,087,177	4.44*at 5%
	SLxB	2,719,334	2	1,359,667	1.96 NS
	DLxB	46,529,619	2	23,264,809	33.49***
	IAAxB	64,331,214	8	8,041,401	11.58***
Second order Interaction	SLxDLxIAA	5,554,593	4	1,388,648	2.00 NS
	SLxDLxB	9,885,578	2	4,942,789	7.12*at 2.5%
	SLxIAAxB	5,695,711	8	711,963	1.03 NS
	DLxIAAxB	52,666,267	8	6,583,283	9.48***
	Error	5,556,695	8	694,586	

DISKS FOUR WEEKS OLD

Main factors	SL	16,912.350	1	16,912,350	12.83*at 1%
	DL	167,090,269	1	167,090,269	126.72***
	IAA	201,858,855	4	50,464,713	38.27***
	B	178,278,776	2	89,139,388	67.60***
First order Interaction	SLxDL	42,762,107	1	42,762,107	32.43***
	SLxIAA	7,727,447	4	1,931,861	1.47 NS
	DLxIAA	19,145,022	4	4,786,255	3.63*at 10%
	SLxB	34,949	2	17,474	NS
	DLxB	44,594,592	2	22,297,296	16.91***
	IAAxB	63,618,051	8	7,952,256	6.03*at 1%
Second order Interaction	SLxDLxIAA	8,348,166	4	2,087,041	1.58 NS
	SLxDLxB	10,574,025	2	5,287,012	4.01*at 10%
	SLxIAAxB	9,850,474	8	1,231,309	NS
	DLxIAAxB	82,833,420	8	10,354,177	7.85***
	Error	10,548,625	8	1,318,578	

TABLE 4 Cont'd.

DISKS FIVE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	9,771,156	1	9,771,156	3.88*at 10%
	DL	195,889,643	1	195,889,643	77.78***
	IAA	224,595,388	4	56,148,847	22.29***
	B	264,592,760	2	132,296,380	52.53***
First order Interaction	SLxDL	44,284,887	1	44,284,887	17.58***
	SLxIAA	16,506,227	4	4,126,556	1.64 NS
	DLxIAA	34,417,844	4	8,604,461	3.33*at 10%
	SLxB	5,639,388	2	2,819,694	1.12 NS
	DLxB	33,993,030	2	16,996,515	6.75*at2.5%
	IAAxB	36,212,436	8	4,526,554	1.80 NS
Second order Interaction	SLxDLxIAA	13,215,508	4	3,303,877	1.31 NS
	SLxDLxB	31,851,969	2	15,925,984	6.32*at2.5%
	SLxIAAxB	13,637,334	8	1,704,666	NS
	DLxIAAxB	62,227,471	8	7,778,433	3.09*at 10%
	Error	20,148,493	8	2,518,561	

DISKS SIX WEEKS OLD

Main factors	SL	10,215,976	1	10,215,976	3.86*at10%
	DL	176,288,328	1	176,288,328	66.67***
	IAA	249,183,265	4	62,295,816	23.56***
	B	300,330,009	2	150,165,004	56.79***
First order Interaction	SLxDL	40,999,361	1	40,999,361	15.51*at1%
	SLxIAA	13,525,744	4	3,381,436	1.28 NS
	DLxIAA	33,721,157	4	8,430,289	3.19*at10%
	SLxB	2,564,473	2	1,282,236	NS
	DLxB	39,589,752	2	19,794,876	7.49*at2.5%
	IAAxB	44,654,001	8	5,581,750	2.11 NS
Second order Interaction	SLxDLxIAA	15,048,734	4	3,762,183	1.42 NS
	SLxDLxB	21,528,729	2	10,764,364	4.07*at10%
	SLxIAAxB	7,460,763	8	932,595	NS
	DLxIAAxB	54,762,572	8	6,845,321	2.59*at10%
	Error	21,154,675	8	2,644,334	

*** Indicates significance at 0.1% level.

SL Seedling-light treatment
DL Disk-light treatment
IAA Concentration of IAA
B Bacterial treatment

TABLE 5. ANALYSIS OF VARIANCE USING ADJUSTED DRY WEIGHTS

DISKS THREE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	25,353,900	1	25,353,900	53.63***
	DL	55,297,920	1	55,297,920	116.96***
	IAA	63,329,565	4	15,832,391	33.49***
	B	53,521,241	2	26,760,620	56.60***
First order Interaction	SLxDL	14,408,940	1	14,408,940	30.48***
	SLxIAA	2,803,883	4	700,970	1.48 NS
	DLxIAA	8,569,767	4	2,142,441	45.32***
	SLxB	4,200,551	2	2,100,275	4.44*at10%
	DLxB	16,991,549	2	8,495,774	17.97***
	IAAxB	31,788,371	8	3,973,546	8.41***
Second order Interaction.	SLxDLxIAA	3,616,563	4	904,140	1.91 NS
	SLxDLxB	1,504,616	2	752,308	1.59 NS
	SLxIAAxB	4,018,157	8	502,269	1.06 NS
	DLxIAAxB	25,040,254	8	3,130,031	6.62*at1%
	Error	3,782,300	8	472,787	

DISKS FOUR WEEKS OLD

Main factors	SL	43,382,306	1	43,382,306	48.37***
	DL	105,695,554	1	105,695,554	117.84***
	IAA	91,572,964	4	22,893,241	25.52***
	B	77,642,984	2	38,821,492	43.28***
First order Interaction	SLxDL	23,878,303	1	23,878,303	26.62***
	SLxIAA	7,868,868	4	1,967,217	2.19 NS
	DLxIAA	10,655,846	4	2,663,961	2.97*at10%
	SLxB	3,334,262	2	1,667,131	1.86 NS
	DLxB	16,315,129	2	8,157,564	9.09*at1%
	IAAxB	28,909,303	8	3,613,662	4.03*at5%
Second order Interaction	SLxDLxIAA	3,005,323	4	751,330	NS
	SLxDLxB	2,674,458	2	1,337,229	1.49 NS
	SLxIAAxB	4,677,890	8	584,736	NS
	DLxIAAxB	38,662,218	8	4,832,777	5.39*at2.5%
	Error	7,175,783	8	896,972	

TABLE 5 Cont'd.

DISKS FIVE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	39,848,610	1	39,848,610	40.15***
	DL	123,229,403	1	123,229,403	124.17***
	IAA	112,822,576	4	28,205,644	28.42***
	B	117,849,631	2	58,924,815	59.37***
First order Interaction	SLxDL	26,264,197	1	26,264,197	26.46***
	SLxIAA	12,181,300	4	3,045,325	3.07*at10%
	DLxIAA	17,751,664	4	4,437,916	4.47*at5%
	SLxB	10,272,167	2	5,136,083	5.18*at5%
	DLxB	13,074,784	2	6,537,392	6.59*at2.5%
	IAAxB	14,965,005	8	1,870,625	1.88 NS
Second order Interaction	SLxDLxIAA	6,908,719	4	1,727,179	1.74 NS
	SLxDLxB	8,722,889	2	4,361,444	4.39*at10%
	SLxIAAxB	5,307,243	8	663,405	NS
	DLxIAAxB	29,330,277	8	3,666,284	3.69*at5%
	Error	7,939,708	8	992,463	

DISKS SIX WEEKS OLD

Main factors	SL	36,890,336	1	36,890,336	33.72***
	DL	113,616,320	1	113,616,320	103.86***
	IAA	123,173,698	4	30,793,424	28.15***
	B	129,179,647	2	64,589,823	59.04***
First order Interaction	SLxDL	18,651,721	1	18,651,721	17.05***
	SLxIAA	12,011,821	4	3,002,955	2.74 NS
	DLxIAA	15,933,952	4	3,983,488	3.64*at10%
	SLxB	6,988,545	2	3,494,272	3.19*at10%
	DLxB	15,331,729	2	7,665,864	7.01*at2.5%
	IAAxB	19,752,496	8	2,469,062	2.26 NS
Second order Interaction	SLxDLxIAA	4,908,280	4	1,227,070	1.12 NS
	SLxDLxB	4,652,500	2	2,326,250	2.13 NS
	SLxIAAxB	2,668,627	8	333,578	NS
	DLxIAAxB	27,427,395	8	3,428,424	3.13*at10%
	Error	8,751,862	8	1,093,982	

TABLE 6. ANALYSIS OF VARIANCE USING ADJUSTED FRESH WEIGHTS BUT OMITTING THE A. TUMEFACIENS TREATMENT

DISKS THREE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	10,080,160	1	10,080,160	9.02*at5%
	DL	115,872,160	1	115,872,160	103.71***
	IAA	146,230,577	4	36,557,644	32.72***
	B	73,240,597	1	73,240,597	65.55***
First order Interaction	SLxDL	26,755,145	1	26,755,145	23.95*at1%
	SLxIAA	3,044,129	4	761,032	NS
	DLxIAA	21,108,677	4	5,277,169	4.72*at10%
	SLxB	1,021,441	1	1,021,441	NS
	DLxB	19,555,225	1	19,555,225	17.50*at2.5%
	IAAxB	46,409,850	4	11,602,462	10.39*at2.5%
Second order Interaction	SLxDLxIAA	5,566,814	4	1,391,703	1.25 NS
	SLxDLxB	8,451,126	1	8,451,126	7.56*at10%
	SLxIAAxB	4,983,507	4	1,245,876	1.12 NS
	DLxIAAxB	42,958,566	4	10,739,641	9.61*at2.5%
	Error	4,469,036	4	1,117,259	

DISKS FOUR WEEKS OLD

Main factors	SL	11,803,736	1	11,803,736	24.06**at1%
	DL	187,510,650	1	187,510,650	382.27***
	IAA	187,054,161	4	46,763,540	95.34***
	B	110,061,380	1	110,061,380	224.38***
First order Interaction	SLxDL	37,475,153	1	37,475,153	76.40***
	SLxIAA	2,239,702	4	559,925	1.14 NS
	DLxIAA	23,950,598	4	5,987,649	12.21*at2.5%
	SLxB	16,769	1	16,769	NS
	DLxB	15,031,986	1	15,031,986	30.65*at1%
	IAAxB	45,263,023	4	11,315,755	23.07*at1%
Second order Interaction	SLxDLxIAA	10,935,568	4	2,733,892	5.57*at10%
	SLxDLxB	8,737,575	1	8,737,575	17.81*at2.5%
	SLxIAAxB	7,767,221	4	1,941,805	3.96NS
	DLxIAAxB	66,009,627	4	16,502,406	33.64***
	Error	1,962,070	4	490,517	

TABLE 6 Cont'd. DISKS FIVE WEEKS OLD

NATURE OF EFFECT	SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F.
Main factors	SL	5,425,059	1	5,425,059	11.66*at5%
	DL	201,605,490	1	201,605,490	433.17***
	IAA	200,715,919	4	50,178,979	107.81***
	B	178,518,925	1	178,518,925	383.56***
First order Interaction	SLxDL	34,731,913	1	34,731,913	74.62***
	SLxIAA	13,526,493	4	3,381,623	7.27*at5%
	DLxIAA	37,010,697	4	9,252,674	19.88*at1%
	SLxB	5,490,069	1	5,490,069	11.80*at5%
	DLxB	10,956,855	1	10,956,855	23.54*at1%
	IAAxB	21,684,856	4	5,421,214	11.65*at2.5%
Second order Interaction	SLxDLxIAA	14,291,998	4	3,572,999	7.68*at5%
	SLxDLxB	31,217,590	1	31,217,590	67.07***
	SLxIAAxB	2,207,890	4	551,972	1.19 NS
	DLxIAAxB	49,981,666	4	12,495,416	26.85***
	Error	1,861,699	4	465,424	

DISKS SIX WEEKS OLD

Main factors	SL	4,846,248	1	4,846,248	4.21 NS
	DL	181,778,586	1	181,778,586	157.84***
	IAA	237,801,830	4	59,450,457	51.62***
	B	201,668,355	1	201,668,355	175.1***
First order Interaction	SLxDL	28,215,600	1	28,215,600	24.50*at1%
	SLxIAA	5,708,183	4	1,427,045	1.24NS
	DLxIAA	36,514,153	4	9,128,538	7.93*at5%
	SLxB	2,064,340	1	2,064,340	1.79 NS
	DLxB	18,655,462	1	18,655,462	16.20*at2.5%
	IAAxB	15,792,342	4	3,948,085	3.43 NS
Second order Interaction	SLxDLxIAA	16,384,490	4	4,096,122	3.56 NS
	SLxDLxB	21,507,690	1	21,507,690	18.67*at2.5%
	SLxIAAxB	4,604,362	4	1,166,096	1.01 NS
	DLxIAAxB	43,114,040	4	10,778,510	9.36*at5%
	Error	4,606,802	4	1,151,700	

TABLE 7. A COMPARISON OF THE LEVELS OF SIGNIFICANCE FOR DIFFERENT TREATMENTS OVER THE FOUR WEEKS OF HARVESTING.

	ALL TREATMENTS ADJUSTED FRESH WEIGHTS				TWO BACTERIAL TREATMENTS ADJUSTED FRESH WEIGHTS				ALL TREATMENTS ADJUSTED DRY WEIGHTS			
SOURCE	3 wks.	4 wks.	5 wks.	6 wks.	3 wks.	4 wks.	5 wks.	6 wks.	3 wks.	4 wks.	5 wks.	6 wks.
SL	12.67*1%	12.83*1%	3.88*10%	3.86*10%	9.02*5%	24.06*1%	11.66*5%	4.21NS	53.63***	48.37***	40.15***	46.38***
DL	130.24***	126.72***	77.78***	66.67***	103.71***	382.27***	433.17***	157.84***	116.96***	117.84***	124.17***	198.91***
IAA	51.13***	38.27***	22.29***	23.56***	32.72***	95.34***	107.81***	51.62***	33.49***	25.52***	28.42***	52.03***
B	96.43***	67.60***	52.53***	56.79***	65.55***	224.38***	383.56***	175.1***	56.60***	43.28***	59.37***	147.20***
SLxDL	43.36***	32.43***	17.58***	15.51***	23.95*1%	76.40***	74.62***	24.50*1%	30.48***	26.62***	26.46***	22.94*1%
SLxIAA	1.33NS	1.47NS	1.64NS	1.28NS	NS	1.14NS	7.27*5%	1.24NS	1.48NS	2.19NS	3.07*10%	3.18NS
DLxIAA	4.44*5%	3.63*10%	3.33*10%	3.19*10%	4.72*10%	12.21*2.5%	19.88*1%	7.93*5%	45.32***	2.97*10%	4.47*5%	8.29*5%
SLxB	1.96NS	NS	1.12NS	NS	NS	NS	11.80*5%	1.79NS	4.44*10%	1.86NS	5.18*5%	11.74*5%
DLxB	33.49***	16.91***	6.75*2.5%	7.49*2.5%	17.50*2.5%	30.65*1%	23.54*1%	16.20*2.5%	17.97***	9.09*1%	6.59*2.5%	4.65*10%
IAAxB	11.58***	6.03*1%	1.80NS	2.11NS	10.39*2.5%	23.07*1%	11.65*2.5%	3.43NS	8.41***	4.03*5%	1.88NS	1.93NS
SLxDLxIAA	2.00NS	1.58NS	1.31NS	1.42NS	1.25NS	5.57*10%	7.68*5%	3.56NS	1.91NS	NS	1.74NS	2.04NS
SLxDLxB	7.12*2.5%	4.01*10%	6.32*2.5%	4.07*10%	7.56*10%	17.81*2.5%	67.07***	18.67*2.5%	1.59NS	1.49NS	4.39*10%	7.92*5%
SLxIAAxB	1.03NS	NS	NS	NS	1.12NS	3.96NS	1.19NS	1.01NS	1.06NS	NS	NS	NS
DLxIAAxB	9.48***	7.85***	3.09*10%	2.59*10%	9.61*2.5%	33.64***	26.85***	9.36*5%	6.62*1%	5.39*2.5%	3.69*5%	8.54*5%

TABLE 8 RATIO OF AVERAGE DRY WEIGHT/INITIAL DRY WEIGHT TO AVERAGE FRESH WEIGHT/INITIAL FRESH WEIGHT

IAA (PPM)	BACTERIAL TREATMENT	SEEDLING AND DISKS - LIGHT TREATMENTS			
		SEEDS AND DISKS-LIGHT	SEEDS-DARK DISKS-LIGHT	SEEDS-LIGHT DISKS-DARK	SEEDS AND DISKS-DARK
0	Uninfected	0.6194	0.7809	0.4932	0.8300
	E. coli	0.6121	0.7561	0.4995	0.8493
	A. tumefaciens	0.5492	0.7632	0.4844	0.7438
0.01	Uninfected	0.6303	0.7729	0.5440	0.8429
	E. coli	0.5899	0.7700	0.5502	0.8010
	A. tumefaciens	0.5720	0.7305	0.4809	0.7246
0.1	Uninfected	0.6064	0.7622	0.4524	0.6618
	E. coli	0.5958	0.7917	0.4458	0.6468
	A. tumefaciens	0.5963	0.8015	0.4510	0.7039
1.0	Uninfected	0.5645	0.7333	0.4505	0.7044
	E. coli	0.5719	0.6926	0.4048	0.5703
	A. tumefaciens	0.6353	0.8109	0.4710	0.6940
10	Uninfected	0.6313	0.7164	0.4483	0.6580
	E. coli	0.6171	0.7736	0.4967	0.6440
	A. tumefaciens	0.6292	0.8270	0.4925	0.6457
	INITIAL DW/FW	0.114	0.088	0.143	0.094

APPENDIX IV

TABLES AND CALCULATIONS FOR CHAPTER SIX

EXPERIMENT 10

TABLE 1. FRESHWEIGHTS OF DISKS GROWN WITH APICAL OR RADICAL END IN CONTACT WITH THE MEDIUM (g)

BACTERIAL TREATMENT	IAA CONCENTRATION		DISK LIGHT TREATMENT: LIGHT — END IN CONTACT WITH MEDIUM: RADICAL	LIGHT — END IN CONTACT WITH MEDIUM: APICAL	DARK — END IN CONTACT WITH MEDIUM: RADICAL	DARK — END IN CONTACT WITH MEDIUM: APICAL
Sterile medium	No IAA		0.5360	0.2970	0.3405	0.2696
			0.3189	0.2293	0.3102	1.6425
			0.3214	0.2771	0.9862	1.9908
			0.2823	0.2202	2.3083	1.9412
			0.3265	0.2392	1.1979	2.3113
			2.3323	1.5658	0.6179	1.8025
			0.3860	2.7683	0.9231	1.6488
			0.2733	2.7051	0.7732	0.9732
			0.2610	2.8585	1.8270	1.2389
			0.2769	1.6930	2.1159	1.7554
		Average	0.5315	1.2854	1.1400	1.5574
E. coli	No IAA		3.4030	3.7111	0.3849	2.1973
			3.3910	3.5828	1.2698	2.0231
			3.8763	0.5412	0.6463	0.7351
			3.5790	3.4195	0.3934	1.3494
			1.8473	3.6732	1.9869	0.3950
			3.0897	3.8492	1.6693	0.3926
			2.6077	3.0764	1.8228	1.3470
			2.2044	2.5473	1.2406	0.8145
			2.9425	3.3931	0.2693	0.5259
			4.5735	0.7610	1.8977	0.9885
		Average	3.1514	2.8555	1.1581	1.0768
Sterile medium	0.01 ppm IAA		4.3318	3.7943	2.3971	2.6787
			4.3990	3.6157	2.5075	1.8242
			3.5513	2.9905	2.0149	1.5280
			2.5706	4.7466	1.9884	1.7122
			3.7014	4.1673	2.2946	2.0844
			4.5033	4.7153	2.2915	2.3933
			3.6265	3.9441	2.9085	1.8023
			3.2316	3.9210	2.9654	2.2000
			3.7486	3.5851	2.1660	1.4938
			3.5407	4.9778	2.3134	1.8047
		Average	3.7205	4.0458	2.3847	1.9522

TABLE 1 Cont'd.

BACTERIAL TREATMENT	IAA CONCENTRATION		DISK LIGHT TREATMENT			
			LIGHT		DARK	
			END IN CONTACT WITH MEDIUM			
			RADICAL	APICAL	RADICAL	APICAL
E. coli	0.01 ppm IAA		4.7737	4.2753	0.2366	0.3060
			4.8996	4.9749	0.3575	0.4013
			4.9639	4.7493	0.2054	0.3505
			4.5641	4.5983	0.2111	0.4238
			3.4306	3.7103	0.3646	0.3141
			4.0862	3.7235	0.3925	0.6186
			4.0329	5.1365	0.5971	0.4162
			3.3262	4.3264	0.7235	0.6899
			4.9916	4.6480	0.6980	0.7319
			5.4431	4.1112	1.4902	1.3398
		Average	4.4512	4.4254	0.5277	0.5592
Synthetic medium	0.1 ppm IAA		5.8196	4.2248	2.2583	1.6756
			4.7208	2.411	2.5416	3.5189
			5.3592	5.0531	2.8249	2.5235
			4.5734	5.0783	2.9143	2.6426
			4.9549	4.1210	2.3220	2.5050
			5.0925	5.3194	2.6979	2.8417
			4.8250	4.7780	2.6059	2.7928
			4.1121	4.8233	2.5240	3.2303
			4.8978	4.8669	1.9538	3.5542
			3.7078	4.4314	2.1126	2.7098
		Average	4.8063	4.5167	2.4755	2.7994
E. coli	0.1 ppm IAA		3.7148	3.7492	0.1633	0.1759
			4.0733	3.6116	0.2525	0.1193
			4.5574	3.5355	0.2543	0.1720
			4.2403	3.4840	0.1507	0.1971
			4.7323	2.1777	0.2000	0.1292
			4.6325	2.7707	0.1454	0.1778
			4.7840	3.1220	0.1785	0.1681
			4.1163	2.0036	0.1818	0.2065
			0.2810	4.0641	0.1397	0.2025
			0.6560	0.1288	0.1455	0.0971
		Average	3.5788	2.8647	0.1812	0.1646

TABLE 1 Cont'd.

BACTERIAL TREATMENT	IAA CONCENTRATION		DISK LIGHT TREATMENT LIGHT		DARK	
			END IN CONTACT WITH MEDIUM			
			RADICAL	APICAL	RADICAL	APICAL
Synthetic medium	1.0 ppm IAA		2.7133	3.3527	1.3105	1.0136
			2.8814	4.3303	1.4567	1.8473
			3.1868	4.2642	1.4189	1.4977
			4.2489	3.5416	1.7333	1.4725
			4.0684	5.0837	0.9717	1.5251
			2.3866	4.2088	1.3966	2.0905
			4.9490	4.2854	1.9251	1.0326
			4.7749	4.6380	1.7483	2.1562
			2.8550	3.9265	1.6312	2.0512
			3.4391	4.9242	2.1315	2.6442
		Average	3.5503	4.2555	1.5724	1.7331
E. coli	1.0 ppm IAA		0.1372	0.1652	0.1039	0.0964
			0.1903	0.1693	0.0590	0.0884
			0.1320	0.2012	0.1204	0.1255
			0.1694	0.1027	0.1391	0.0955
			0.1275	0.1748	0.1138	0.1518
			0.1972	0.1296	0.1192	0.1349
			0.2248	0.2555	0.0800	0.1061
			0.8551	0.0958	0.0905	0.0766
			0.8841	0.4963	0.1306	0.1368
			1.3164	2.2529	0.1243	0.1250
		Average	0.4234	0.4043	0.1081	0.1137

DETAILS OF SIGNIFICANCE TESTS

Light - Uninfected disks grown without IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	283,449,405	283,449,405	3.202*
Among apical + among basal	9+9	1,593,427,832	88,523,768	at 10%
Total	19			

Light - Disks inoculated with E. coli and grown without IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	43,778,405	43,778,405	N.S.
Among apical + among basal	9+9	2,020,284,066	112,238,004	
Total	19			

Light - Uninfected disks grown with 0.01 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	52,910,045	52,910,045	1.465
Among apical + among basal	9+9	650,186,200	36,121,456	N.S.
Total	19			

Light - Disks inoculated with E. coli and grown with 0.01 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	332,820	332,820	N.S.
Among apical + among basal	9+9	659,907,763	36,661,542	
Total	19			

Light - Uninfected disks grown with 0.1 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	41,934,080	41,934,080	N.S.
Among apical + among basal	9+9	916,126,583	50,595,921	
Total	19			

Light - Disks inoculated with E. coli and grown with 0.1 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	254,969,405	254,969,405	1.219
Among apical + among basal	9+9	3,764,468,603	209,137,145	N.S.
Total	19			

Light - Uninfected disks grown with 1.0 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	248,653,520	248,653,520	4.447*
Among apical + among basal	9+9	1,006,367,892	55,909,327	at 5%
Total	19			

Light - Disks inoculated with E. coli and grown with 1.0 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	163,805	163,805	N.S.
Among apical + among basal	9+9	557,573,556	30,976,309	
Total	19			

Dark - Uninfected disks grown without IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	87,111,380	87,111,380	2.032
Among apical + among basal	9+9	771,776,402	42,876,467	N.S.
Total	19			

Dark - Disks inoculated with E. coli and grown without IAA.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	3,304,845	3,304,845	N.S.
Among apical + among basal	9+9	791,901,276	43,994,515	
Total	19			

Dark - Uninfected disks grown with 0.01 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	93,528,125	93,528,125	7.323*
Among apical + among basal	9+9	229,887,546	12,771,530	at 2.5%
Total	19			

Dark - Disks inoculated with E. coli and grown with 0.01 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	496,125	496,125	N.S.
Among apical + among basal	9+9	225,249,007	12,513,833	
Total	19			

Dark - Uninfected disks grown with 0.1 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	52,455,605	52,455,605	2.653
Among apical + among basal	9+9	359,566,250	19,975,903	N.S.
Total	19			

Dark - Disks inoculated with E. coli and grown with 0.1 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	137,780	137,780	N.S.
Among apical + among basal	9+9	2,879,610	159,978	
Total	19			

Dark - Uninfected disks grown with 1.0 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	12,912,245	12,912,245	N.S.
Among apical + among basal	9+9	342,061,449	19,003,414	
Total	19			

Dark - Disks inoculated with *E. coli* and grown with 1.0 ppm IAA

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Apical vs basal orientation	1	15,680	15,680	N.S.
Among apical + among basal	9+9	1,097,088	60,949	
Total	19			

EXPERIMENT 12

TABLE 2. FRESH WEIGHTS OF DISKS INFECTED AT VARYING TIMES AFTER PREPARATION (g)

TIME BETWEEN ISOLATION AND INOCULATION	DISK LIGHT TREATMENT	
	LIGHT	DARK
Uninfected disks	0.2900	0.8067
	0.4212	0.3580
	0.2069	1.3334
	0.2573	0.3775
	0.2172	0.8591
	0.1990	0.4463
	0.3113	0.7374
	0.2648	0.3911
	0.3858	0.4516
	1.6056	0.3185
Average	0.4159	0.6080
Disks inoculated with E. coli immediately (0 hours)	3.0191	1.7520
	2.0547	0.2078
	2.2153	0.4904
	2.8762	0.5544
	0.3613	0.3294
	3.6058	0.2211
	3.2044	0.3138
	4.0896	1.4230
	0.6004	0.5370
		0.2995
Average	2.4475	0.6128
12 hours	3.1088	1.8816
	2.5481	0.3569
	1.8907	0.3965
	0.6148	1.1454
	1.7415	1.9748
	2.4665	1.0694
	3.7710	1.2499
	0.5225	1.1469
	1.6478	1.3985
	4.1144	1.0049
Average	2.2426	1.1625

TABLE 2. Cont'd.

TIME BETWEEN ISOLATION AND INOCULATION	DISK LIGHT TREATMENT	
	LIGHT	DARK
24 hours	0.3705	2.1431
	3.4942	0.9483
	1.0289	2.0550
	0.2525	0.8268
	0.2352	1.2990
	0.3777	1.4928
	0.3714	0.4450
	0.3103	0.6878
	3.1040	1.6316
	0.3432	2.2597
Average	0.9888	1.3789
36 hours	0.5945	0.7142
	0.2761	2.2628
	0.3661	1.3003
	0.2086	2.1597
	1.9145	0.6845
	0.3862	2.5066
	1.9340	1.1395
	0.2886	0.8088
	0.3439	2.2106
	2.9213	1.5628
Average	0.9234	1.5350
48 hours	0.2531	2.1567
	0.2885	1.6084
	0.6013	0.3832
	0.6713	1.9376
	0.8983	0.3086
	3.0371	1.5430
	1.9824	0.5933
	2.8465	0.5052
		1.6309
Average	1.3223	1.1852

TABLE 2. Cont'd.

TIME BETWEEN ISOLATION AND INOCULATION	DISK LIGHT TREATMENT	
	LIGHT	DARK
60 hours	2.1048	1.9410
	0.5321	2.3535
	0.3153	1.4296
	3.7564	1.6239
	0.4034	2.6155
	0.8635	0.7545
	0.6577	3.2132
	0.2064	1.7014
	0.2964	1.1732
	4.0819	2.6648
Average	1.3218	1.9470
72 hours	1.1826	1.3359
	0.3147	1.8414
	0.3347	0.9908
	0.3611	1.8128
	0.2851	1.7734
	1.3675	2.2354
	0.1781	1.7092
	0.2293	1.5684
	0.2356	0.7540
	0.3330	1.4106
Average	0.4822	1.5432
84 hours	0.4389	0.8620
	0.5010	1.9647
	0.3834	0.6986
	0.5120	2.6182
	0.7145	1.2932
	2.0198	2.9400
	0.6668	0.8156
	0.5933	1.7903
	0.4404	0.6411
	0.3814	2.0319
Average	0.6652	1.5656

TABLE 2. Cont'd.

TIME BETWEEN ISOLATION AND INOCULATION	DISK LIGHT TREATMENT	
	LIGHT	DARK
96 hours	0.3026	0.3875
	0.3891	1.2140
	2.3621	1.2143
	1.7338	0.6128
	0.2778	1.8675
	2.1811	0.3490
	0.2606	0.3725
	0.2641	2.0594
	0.3120	1.0698
	0.7631	1.3776
Average	0.8846	1.0524
108 hours	0.5273	0.5124
	1.6744	1.8422
	1.8513	1.5436
	0.2674	1.5841
	0.4411	1.9221
	0.4033	1.3126
	0.4031	2.1795
	0.2667	2.3113
	0.3767	0.7650
	0.5399	0.8749
Average	0.6751	1.4848
120 hours	0.5791	0.5808
	0.6257	1.1378
	0.2225	2.0648
	0.3295	0.5332
	0.5032	0.4192
	0.2768	1.2961
	0.7853	1.2897
	1.3837	0.4254
	1.2871	1.0921
	0.3661	0.8740
Average	0.6359	0.9713

DETAILS OF SIGNIFICANCE TESTS

Disks grown in the light. Comparing uninfected disks with those inoculated with E. coli immediately.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-0 hrs.	1	1,954,891,080	1,954,891,080	22.58***
Among treatments	9+8	1,471,508,226	86,559,307	
Total	18			

Light - Comparing uninfected disks with those inoculated with E. coli after 12 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-12 hrs.	1	1,668,416,445	1,668,416,445	20.44***
Among treatments	9+9	1,469,191,004	81,621,722	
Total	19			

Light - Comparing uninfected disks with those inoculated with E. coli after 24 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-24 hrs.	1	164,107,455	164,107,455	1.91
Among treatments	9+9	1,549,814,696	86,100,816	N.S.
Total	19			

Light - Comparing uninfected disks with those inoculated with E.coli after 36 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-36 hrs.	1	128,778,125	128,778,125	2.32
Among treatments	9+9	999,257,157	55,514,287	N.S.
Total	19			

Light - Comparing uninfected disks with those inoculated with E. coli after 48 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-48 hrs.	1	361,486,822	361,486,822	5.43*
Among treatments	9+7	1,065,611,140	66,600,696	at 5%
Total	17			

Light - Comparing uninfected disks with those inoculated with E.coli after 60 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-60 hrs.	1	410,327,405	410,327,405	3.48*
Among treatments	9+9	2,120,658,072	117,814,337	at 10%
Total	19			

Light - Comparing uninfected disks with those inoculated with E.coli after 72 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-72 hrs.	1	2,197,845	2,197,845	N.S.
Among treatments	9+9	323,845,541	17,991,419	
Total	19			

Light - Comparing uninfected disks with those inoculated with E.coli after 84 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-84 hrs.	1	31,075,245	31,075,245	1.48
Among treatments	9+9	377,598,171	20,977,676	N.S.
Total	19			

Light - Comparing uninfected disks with those inoculated with E.coli after 96 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-96 hrs.	1	109,839,845	109,839,845	2.13
Among treatments	9+9	827,584,451	51,532,469	N.S.
Total	19			

Light- Comparing uninfected disks with those inoculated with E.coli after 108 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-108hrs.	1	33,592,320	33,592,320	1.29
Among treatments	9+9	466,814,409	25,934,134	N.S.
Total	19			

Light - Comparing uninfected disks with those inoculated with E.coli after 120 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-120hrs.	1	24,200,000	24,200,000	1.39
Among treatments	9+9	311,435,721	17,301,985	N.S.
Total	19			

Dark - Comparing uninfected disks (using average weights from Experiments 10 and 11) with those inoculated with E.coli immediately.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	112,121,340	112,121,340	5.46*
Among treatments	5+9	287,473,961	20,533,854	at 5%
Total	15			

DARK - Comparing uninfected disks with those inoculated with E.coli after 12 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-12 hrs.	1	3,154	3,154	N.S.
Among treatments	5+9	282,113,301	20,150,951	
Total	15			

Dark - Comparing uninfected disks with those inoculated with E.coli after 24 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-24 hrs.	1	18,034,634	18,034,634	N.S.
Among treatments	5+9	405,122,883	28,937,349	
Total	15			

Dark - Comparing uninfected disks with those inoculated with E.coli after 36 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-36 hrs.	1	52,846,935	52,846,935	1.55
Among treatments	5+9	476,816,501	34,058,322	N.S.
Total	15			

Dark - Comparing uninfected disks with those inoculated with E.coli after 48 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-48 hrs.	1	235,930	235,930	N.S.
Among treatments	5+8	455,113,640	35,008,742	
Total	14			

Dark - Comparing uninfected disks with those inoculated with E.coli after 60 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-60 hrs.	1	232,499,535	232,499,535	5.93*
Among treatments	9+5	549,245,043	39,231,789	5%
Total	15			

Dark- Comparing uninfected disks with those inoculated with E.coli after 72 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-72 hrs.	1	55,180,860	55,180,860	3.82*
Among treatments	9+5	202,154,342	14,439,596	at 10%
Total	15			

Dark - Comparing uninfected disks with those inoculated with E.coli after 84 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-84 hrs.	1	61,813,500	61,813,500	1.34
Among treatments	9+5	647,336,631	46,238,331	N.S.
Total	15			

Dark - Comparing uninfected disks with those inoculated with E.coli after 96 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli-96 hrs.	1	4,309,440	4,309,440	N.S.
Among treatments	9+5	374,027,035	26,716,217	
Total	15			

Dark - Comparing uninfected disks with those inoculated with *E.coli* after 108 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs *E.coli*-108hrs.	1	39,658,140	39,658,140	1.51
Among treatments	5+9	367,437,261	26,245,519	N.S.
Total	15			

Dark - Comparing uninfected disks with those inoculated with *E.coli* after 120 hours.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs *E.coli*-120hrs.	1	13,296,334	13,296,334	N.S.
Among treatments	5+9	271,271,476	19,376,534	
Total	15			

EXPERIMENT 13

TABLE 3. INITIAL FRESH WEIGHTS OF HYPOCOTYL DISKS PREPARED FROM SEEDLINGS OBTAINED FROM TWO SOURCES

1963 Seed Batch	1964 Seed Batch
0.0301	0.0268
0.0236	0.0357
0.0271	0.0255
0.0270	0.0237
0.0215	0.0252
0.0233	
0.0201	
0.0248	
0.0254	
0.0257	
Average 0.0249	0.0274

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
1963 vs 1964	1	3413	3413	2.52
Among seed treatments	9+4	8,478+9,139	1355	NS
Total	14			

TABLE 4. FRESH AND DRY WEIGHTS OF HYPOCOTYL DISKS INOCULATED WITH TWO STRAINS OF E.COLI, AND PREPARED FROM DIFFERENT SEEDS (g)

	1963 Seeds		1964 Seeds	
E. coli Strain I	Fresh	Dry	Fresh	Dry
	1.0600	0.0854	1.0651	0.0797
	0.3045	0.0257	1.3193	0.1006
	0.5028	0.0374	2.0535	0.1493
	0.4287	0.0309	0.2424	0.0169
	0.2848	0.0216	0.2055	0.0151
	0.2836	0.0203	0.5048	0.0383
	0.1980	0.0126	1.0042	0.0807
	0.3588	0.0332	0.6679	0.0473
	0.1388	0.0135	0.2512	0.0188
	1.0574	0.0748	0.4132	0.0303
Average wt.	0.4617	0.0355	0.7727	0.0577
E. coli Strain II	0.2536	0.0233	0.4545	0.0351
	1.1146	0.0852	0.5890	0.0350
	1.5404	0.1274	0.2921	0.0215
	1.3085	0.0955	1.3888	0.1289
	0.6594	0.0476	1.2608	0.1036
	0.4997	0.0437	1.2767	0.1040
	0.1586	0.0127	0.2738	0.0207
	0.2220	0.0174	0.3788	0.0286
	0.7583	0.0584	0.9236	0.0768
	0.7428	0.0585	0.2582	0.0181
Average wt.	0.7258	0.0570	0.7096	0.0572

ANALYSIS OF VARIANCE COMPARING THE STRAINS OF BACTERIA USED, WITH THE DIFFERENT SEEDS

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F
Bacterial strain	1	149,818	149,818	NS
Seed batch	1	157,503	157,503	NS
Bacteria x seed	1	58,271,658	58,271,658	2.61
Error	36	805,214,300	22,367,064	

APPENDIX V

TABLES AND CALCULATIONS FOR CHAPTER SEVEN

EXPERIMENT 14a

TABLE 1. AVERAGE FRESH WEIGHTS* AND STANDARD ERRORS OF TISSUE PIECES FOUR WEEKS OLD (g)

MEDIUM	BACTERIAL TREATMENT	LIGHT TREATMENT OF TISSUES	
		LIGHT	DARK
Skoog's	Uninfected	1.1199 ± 0.2495	0.9056 ± 0.0947
	E. coli	0.0278 ± 0.0016	0.0335 ± 0.0035
+	*A. tumefaciens*	0.3268	0.6519
de Ropp's	Uninfected	0.0326 ± 0.0038	0.0296 ± 0.0016
	E. coli	0.0402 ± 0.0040	0.0377 ± 0.0039
+	*A. tumefaciens*	0.1211	0.0936

* Weight of one piece of tissue.

\+ No standard errors were found for these treatments since each contained only two replicates.

Comparison between uninfected and E. coli-inoculated tissue pieces grown on de Ropp's medium.

a) In the Light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	23,104	23,104	1.898
Among treatments	7+7	170,385	12,170	N.S.
Total	15			

b) In the Dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	26,244	26,244	3.67*
Among treatments	7+7	100,017	7,144	10%
Total	15			

Comparison between E.coli-inoculated tissue pieces grown on Skoog's and de Ropp's medium.

a) In the light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Skoog's vs de Ropp's	1	61,504	61,504	8.25*
Among treatments	7+7	104,332	7,452	at 2.5%
Total	15			

b) In the dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Skoog's vs de Ropp's	1	6,580	6,580	N.S.
Among treatments	7+6	137,825	10,602	
Total	14			

EXPERIMENT 14b

TABLE 2. AVERAGE FRESH WEIGHTS* AND STANDARD ERRORS OF TISSUE PIECES FOUR WEEKS OLD (g)

MEDIUM	BACTERIAL TREATMENT	LIGHT TREATMENT OF TISSUES	
		LIGHT	DARK
Skoog's de Ropp's	*E. coli*	0.0830 ± 0.0033	0.0944 ± 0.0031
	Uninfected	0.0997 ± 0.0036	0.1122 ± 0.0058
	E. coli	0.1221 ± 0.0054	0.1072 ± 0.0037

* Weight of three pieces of tissue.

TABLE 3. DRY WEIGHT/FRESH WEIGHT* AND STANDARD ERRORS OF TISSUE PIECES FOUR WEEKS OLD

MEDIUM	BACTERIAL TREATMENT	LIGHT TREATMENT OF TISSUES	
		LIGHT	DARK
Skoog's de Ropp's	*E. coli*	0.0969 ± 0.0015	0.0884 ± 0.0013
	Uninfected	0.1132 ± 0.0013	0.1009 ± 0.0028
	E. coli	0.1079 ± 0.0019	0.0937 ± 0.0018

* Weight of three pieces of tissue

Comparison between fresh weights of uninfected and E.coli-inoculated tissues grown on de Ropp's medium.

a) In the light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	878,080	878,080	11.96***
Among treatments	34+34	4,992,364	73,417.	
Total	69			

b) In the dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	45,000	45,000	N.S.
Among treatments	35+35	6,026,217	86,089	
Total	71			

Comparison between fresh weights of E.coli-inoculated tissues grown on de Ropp's or Skoog's medium.

a) In the light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
de Ropp's vs Skoog's	1	488,058	488,058	11.66***
Among treatments	34+34	2,847,195	41,871	
Total	69			

b) In the dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
de Ropp's vs Skoog's	1	211,750	211,750	N.S.
Among treatments	35+33	65,946,127	969,796	
Total	69			

A comparison between the dry weight/fresh weight ratio for uninfected and E. coli-inoculated tissues grown on de Ropp's medium.

a) In the light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	49,158	49,158	N.S.
Among treatments	34+34	622,919	91,606	
Total	69			

b) In the dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Uninfected vs E.coli	1	93,312	93,312	4.74*
Among treatments	35+35	1,379,421	19,706	5%
Total	71			

A comparison between the dry weight/fresh weight ratio for E.coli-inoculated tissues grown on de Ropp's or Skoog's medium.

a) In the light.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Skoog's vs de Ropp's	1	211,750	211,750	20.99***
Among treatments	34+34	685,990	10,088	
Total	69			

b) In the dark.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F.
Skoog's vs de Ropp's	1	49,129	49,129	5.67*
Among treatments	35+33	589,790	8,673	at 2.5%
Total	69			

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